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(71) Applicant: **RESPONSE GENETICS, INC.** [US/US];
1640 Marengo Street, 6th Floor, Los Angeles, CA 90033
(US).

(72) Inventor: **DANENBERG, Kathleen, D.**; 3367 Rubio
Crest Drive, Altadena, CA 91001 (US).

(74) Agents: **MELORO, Thomas, J.** et al.; Kenyon & Kenyon,
1500 K Street, N.W., Suite 700, Washington, DC 20005
(US).

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(54) Title: METHOD OF DETERMINING EPIDERMAL GROWTH FACTOR RECEPTOR AND HER2-NEU GENE EXPRESSION AND CORRELATION OF LEVELS THEREOF WITH SURVIVAL RATES

(57) Abstract: The present invention relates to prognostic methods which are useful in medicine, particularly cancer chemotherapy. The object of the invention to provide a method for assessing *HER2-neu* and/or *EGFR* expression levels in fixed or fixed and paraffin embedded tissues and prognosticate the probable sensitivity of a patient's tumor to treatment with receptor tyrosine kinase targeted chemotherapy by examination of the amount of *HER2-neu* and/or *EGFR* mRNA in a patient's tumor cells and comparing it to a predetermined threshold expression level for those genes. More specifically, the invention provides to oligonucleotide primer pairs *EGFR* and *HER2-neu* and methods comprising their use for detecting levels of *EGFR* and *HER2-neu*mRNA, respectively.



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**METHOD OF DETERMINING *EPIDERMAL GROWTH FACTOR*
RECEPTOR AND *HER2-neu* GENE EXPRESSION AND
CORRELATION OF LEVELS THEREOF WITH SURVIVAL
RATES**

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FIELD OF THE INVENTION

The present invention relates to prognostic methods which are useful in medicine, particularly cancer chemotherapy. More particularly, the invention relates to assessment of survivability of a patient whose tumor cell gene expression
10 is analyzed. Additionally, the sensitivity of tumor cells to receptor tyrosine kinase targeted chemotherapeutic regimen is assayed by examining the mRNA expression of the *EGFR* and *Her2-neu* genes in humans.

BACKGROUND OF THE INVENTION

Lung cancer is the leading cause of cancer-related deaths among both males
15 and females in western countries. In the United States, approximately 171,000 new cases of lung cancer are diagnosed and 160,000 individuals die from this disease each year. Despite improvements in the detection and treatment of lung cancer in the past two decades, the overall 5-year survival remains less than 15% . Ginsberg, et al., In: DeVita, et al., Cancer: Principles in Practice of Oncology, Ed. 5, pp. 858-
20 910. Philadelphia: Lipincott-Raven Publishers, 1997. To further improve the survival rate in patients with Non-Small Cell Lung Carcinoma (NSCLC), their prognostic classification based on molecular alterations is crucial. Such

classification will provide more accurate and useful diagnostic tools and, eventually, more effective therapeutic options.

Cancer arises when a normal cell undergoes neoplastic transformation and becomes a malignant cell. Transformed (malignant) cells escape normal physiologic controls specifying cell phenotype and restraining cell proliferation. Transformed
5 cells in an individual's body thus proliferate, forming a tumor. When a tumor is found, the clinical objective is to destroy malignant cells selectively while mitigating any harm caused to normal cells in the individual undergoing treatment.

Chemotherapy is based on the use of drugs that are selectively toxic
10 (cytotoxic) to cancer cells. Several general classes of chemotherapeutic drugs have been developed, including drugs that interfere with nucleic acid synthesis, protein synthesis, and other vital metabolic processes. These generally are referred to as anti-metabolite drugs. Other classes of chemotherapeutic drugs inflict damage on cellular DNA. Drugs of these classes generally are referred to as genotoxic.
15 Additionally, a class of chemotherapeutic agents specifically inhibit mitogenic signaling through receptor tyrosine kinases (RTKs), in cells where RTKs are over active. (Drugs of the Future, 1992, 17, 119).

Susceptibility of an individual neoplasm to a desired chemotherapeutic drug or combination of drugs often, however, can be accurately assessed only after a trial
20 period of treatment. The time invested in an unsuccessful trial period poses a significant risk in the clinical management of aggressive malignancies. Therefore, it is of importance to assess the expression status of genetic determinants targeted by specific chemotherapeutic agents. For example, if a tumor expresses high levels of DNA repair genes, it is likely that the tumor will not respond well to low doses of
25 DNA-damaging genotoxic agents. Thus, the expression status of genetic

determinants of a tumor will help the clinician develop an appropriate chemotherapeutic regimen specific to the genetic repertoire of the tumor.

Receptor tyrosine kinases (RTKs) are important in the transduction of mitogenic signals. RTKs are large membrane spanning proteins which possess an extracellular ligand binding domain for growth factors such as epidermal growth factor (EGF) and an intracellular portion which functions as a kinase to phosphorylate tyrosine amino acid residues on cytosol proteins thereby mediating cell proliferation. Various classes of receptor tyrosine kinases are known based on families of growth factors which bind to different receptor tyrosine kinases. (Wilks, Advances in Cancer Research, 1993, 60, 43-73)

Class I kinases such as the EGF-R family of receptor tyrosine kinases include the EGF, HER2-neu, erbB, Xmrk, DER and let23 receptors. These receptors are frequently present in common human cancers such as breast cancer (Sainsbury et al., Brit. J. Cancer, 1988, 58, 458; Guerin et al., Oncogene Res., 1988, 3, 21), squamous cell cancer of the lung (Hendler et al., Cancer Cells, 1989, 7, 347), bladder cancer (Neal et al., Lancet, 1985, 366), oesophageal cancer (Mukaida et al., Cancer, 1991, 68, 142), gastrointestinal cancer such as colon, rectal or stomach cancer (Bolen et al., Oncogene Res., 1987, 1, 149), leukaemia (Konaka et al., Cell, 1984, 37, 1035) and ovarian, bronchial or pancreatic cancer (European Patent Specification No. 0400586). As further human tumor tissues are tested for the EGF family of receptor tyrosine kinases it is expected that its widespread prevalence will be established in other cancers such as thyroid and uterine cancer.

Specifically, EGFR tyrosine kinase activity is rarely detected in normal cells whereas it is more frequently detectable in malignant cells (Hunter, Cell, 1987, 50, 823). It has been more recently shown that *EGFR* is overexpressed in many human

cancers such as brain, lung squamous cell, bladder, gastric, breast, head and neck, oesophageal, gynaecological and thyroid tumours. (W J Gullick, Brit. Med. Bull., 1991, 47, 87). Receptor tyrosine kinases are also important in other cell-proliferation diseases such as psoriasis. EGFR disorders are those characterized
5 by *EGFR* expression by cells normally not expressing *EGFR*, or increased EGFR activation leading to unwanted cell proliferation, and/or the existence of inappropriate *EGFR* levels. The EGFR is known to be activated by its ligand EGF as well as transforming growth factor-alpha (TGF- α).

The Her2-neu protein is also a member of the class I receptor tyrosine
10 kinase (RTK) family. Yarden and Ullrich, Annu. Rev. Biochem. 57:443, 1988; Ullrich and Schlessinger, Cell 61:203, 1990. Her2-neu protein is structurally related to EGFR. Carraway, et al., Cell 78:5, 1994; Carraway, et al., J. Biol. Chem. 269:14303, 1994. These receptors share a common molecular architecture and contain two cysteine-rich regions within their cytoplasmic domains and structurally
15 related enzymatic regions within their cytoplasmic domains.

Ligand-dependent activation of Her2-neu protein is thought to be mediated by neuactivating factor (NAF) which can directly bind to p165(Her2-neu) and stimulate enzymatic activity. Dougall et al., Oncogene 9:2109, 1994; Samata et al., Proc. Natl. Acad. Sci. USA 91:1711, 1994. Ligand-independent homodimerization
20 of Her2-neu protein and resulting receptor activation is facilitated by over-expression of Her2-neu protein. An activated Her2-neu complex acts as a phosphokinase and phosphorylates different cytoplasmic proteins. HER2-neu disorders are characterized by inappropriate activity or over-activity of HER2-neu have increased HER2-neu expression leading to unwanted cell proliferation such as
25 cancer.

Inhibitors of receptor tyrosine kinases EGFR and HER2-neu are employed as selective inhibitors of the growth of mammalian cancer cells (Yaish et al. Science, 1988, 242, 933). For example, erbstatin, an EGF receptor tyrosine kinase inhibitor, reduced the growth of EGFR expressing human mammary carcinoma cells
5 injected into athymic nude mice, yet had no effect on the growth of tumors not expressing EGFR. (Toi et al., Eur. J. Cancer Clin. Oncol., 1990, 26, 722.) Various derivatives of styrene are also stated to possess tyrosine kinase inhibitory properties (European Patent Application Nos. 0211363, 0304493 and 0322738) and to be of use as anti-tumour agents. Two such styrene derivatives are Class I RTK inhibitors
10 whose effectiveness has been demonstrated by attenuating the growth of human squamous cell carcinoma injected into nude mice (Yoneda et al., Cancer Research, 1991, 51, 4430). It is also known from European Patent Applications Nos. 0520722 and 0566226 that certain 4-anilinoquinazoline derivatives are useful as inhibitors of receptor tyrosine kinases. The very tight structure-activity relationships shown by
15 these compounds suggests a clearly-defined binding mode, where the quinazoline ring binds in the adenine pocket and the anilino ring binds in an adjacent, unique lipophilic pocket. Three 4-anilinoquinazoline analogues (two reversible and one irreversible inhibitor) have been evaluated clinically as anticancer drugs. Denny, Farmaco 2001 Jan-Feb;56(1-2):51-6. Recently, the U.S. FDA approved the use of
20 the monoclonal antibody trastuzumab (Herceptin®) for the treatment of HER2-neu overexpressing metastatic breast cancers. Scheurle, et al., Anticancer Res 20:2091-2096, 2000.

Because effective chemotherapy against tumors often requires a combination of agents, the identification and quantification of determinants of resistance or
25 sensitivity to each single drug has become an important tool to design individual

combination chemotherapy. Studies have unsuccessfully attempted to reliably correlate the relative levels of expression of *EGFR* and/or *HER2-neu* in malignant cells from cancer patients with survivability.

The prognostic importance of *EGFR* and in NSCLC has heretofore remained
5 controversial. Studies using binding assays correlated increased *EGFR* expression with advanced stage NSCLC and shortened overall survival, whereas studies using semi-quantitative techniques for measuring *EGFR* mRNA or protein expression failed to show a consistent correlation with clinical outcome. Veale et al., Br. J. Cancer 68:162-165, 1993; Fujino et al., Eur. Cancer 32:2070-2074, 1996; Rusch, et
10 al., Cancer Res 53:2379-2385, 1993; Pfeiffer, et al., Br J Cancer 74:86-91, 1996; Pastorino, et al., J Clin Oncol 15:2858-2865, 1997. Studies of *EGFR* expression in NSCLC tumors using immunohistochemical methods have shown frequencies for *EGFR* overexpression between 32% and 47% in NSCLC tumors. Veale et al., Br. J. Cancer 55:513-516, 1987; Veale et al., Br. J. Cancer 68:162-165, 1993; Fujino et al.,
15 Eur. Cancer 32:2070-2074, 1996; Rusch, et al., Cancer Res 53:2379-2385, 1993; Pastorino et al., J.Clin.Onc. 15:2858-2865, 1997; Tateishi, et al., Eur J Cancer 27:1372-75, 1991; Rachwal, et al., Br J Cancer 72:56-64, 1995; Rusch, et al., Cancer Res 15:2379-85, 1993; Pfeiffer, et al., Br J Cancer 78:96-9, 1998; Ohsaki, et al., Oncol Rep 7:603-7, 2000. Moreover, significant differences in *EGFR* expression
20 has been reported among histological subtypes, generally with higher *EGFR* expression in SCC compared to AC and LC. Fujino et al., Eur. Cancer 32:2070-2074, 1996; Veale et al., Br. J. Cancer 55:513-516, 1987; Pastorino et al., J.Clin.Onc. 15:2858-2865, 1997; Pfeiffer, et al., Br J Cancer 78:96-9, 1998; Ohsaki et al., Oncol. Rep. &:603-7, 2000. However, these studies reported no consistent
25 correlation of *EGFR* overexpression with lung cancer patient survival.

Observations of a purported correlation of *EGFR* overexpression with a decrease in patient survival were made in some inconclusive studies. Veale et al., 1987; Ohsaki et al., 2000. However, Veale et al., analyzed a population of only nineteen NSCLC patients. Ohsaki et al., correlated *EGFR* protein expression with poor prognosis in NSCLC patients with p53 overexpression ($P=0.024$).

As with *EGFR*, the prognostic importance of *HER2-neu* and in NSCLC has heretofore remained controversial. *HER2-neu* protein overexpression has been demonstrated in NSCLC, including squamous cell carcinoma, adenocarcinoma, and large cell carcinoma. Veale et al., 1987; Schneider, et al., *Cancer Res* 49:4968-4971, 1989; Kern et al., *Cancer Res* 50:5184-5191, 1990; Weiner, et al., *Cancer Res* 50:421-425, 1990; Scheurle, et al., *Anticancer Res* 20:2091-2096, 2000. Earlier studies, using protein assays, reported an association of *HER2-neu* protein overexpression and inferior overall survival in pulmonary adenocarcinomas (AC). Kern, et al., *Cancer Res* 50:5184-5191, 1990; Kern et al., *J Clin Invest* 93:516-20, 1994. However, contradictory studies reported no correlation of *HER2-neu* protein overexpression with inferior overall survival in pulmonary adenocarcinomas (AC). Pfeiffer et al., *Br. J. Cancer* 74:86-91, 1996.

Another critical question is the evaluation of interrelationships between *HER2-neu* and *EGFR* co-overexpression as prognosticators of cancer. Tateishi et al., (*Eur. J. Cancer* 27:1372-75, 1991), measured *EGFR* and *HER2-neu* protein co-expression, in 13% of AC analysed, and found that co-overexpression of these two genes correlated with inferior 5-year survival. However, as with *HER2-neu* overexpression alone, association between *HER2-neu* and *EGFR* co-expression and survival in squamous cell carcinoma (SCC) and large cell carcinoma (LCC) of the lung has not been reported.

Inconsistent methodologies for the determination of *EGFR* and *HER2-neu* expression levels has been at the root of the problem in determining to what extent expression of these genes may be used to prognosticate cancer patient survivability. Heretofore investigations of *HER2-neu* and *EGFR* expression in NSCLC has

5 resulted in enormous variations in frequencies of NSCLC tumors scored positive for both *EGFR* and *HER2-neu* expression. Overexpression of *HER2-neu*, defined as positive protein staining in adenocarcinomas (AC), was reported in 13-80%, in 2-45% in squamous cell carcinomas (SCC), and in 0-20% in large cell carcinomas (LC) by using paraffin embedded tissue on light microscope slides and *HER2-neu*

10 antisera. Pfeiffer et al., 1996; Kern et al., 1990; Kern et al., 1994; Tateishi et al., 1991; Shi, et al., Mol Carcing 5:213-8, 1992; Bongiorno, et al., J Thorac Cardiovasc Surg 107:590-5,1994; Harpole, et al., Clin Cancer Res 1:659-64, 1995; Volm et al., Anticancer Res 12:11-20,1992. Moreover, a recent report illustrates the non-specificity of current protocols designed to assess *HER2-neu* expression levels.

15 The HercepTest® for measurement of *HER2-neu* expression in invasive breast cancers was shown to have very high false positivity. Jacobs et al., J Clin Oncol 17:1983-1987, 1999.

If a precise, accurate, and consistent method for determining the expression levels of *EGFR* and *HER2-neu* existed, one could ascertain what expression levels

20 correlate to patient survivability and whether or not a receptor tyrosine kinase targeted chemotherapy is appropriate. Consistent demonstration of *EGFR* and/or *HER2-neu* overexpression in NSCLC, using a standardized method, is desirable in establishing clinical trials for current and future receptor tyrosine kinase targeted chemotherapies, e.g., chemotherapeutic agents, antibody-based drugs, to treat

25 cancers overexpressing these receptors.

The current protocols for measuring *EGFR* and/or *HER2-neu* gene expression, aside from being insufficiently accurate for tumor prognostication, suffer from a second limitation in that they require a significant amount of fresh tissue that contains non-degraded mRNA. Most patient derived pathological samples are routinely fixed and paraffin-embedded (FPE) to allow for histological analysis and subsequent archival storage. Thus, most biopsy tissue samples are not useful for analysis of gene expression because such studies require a high integrity of RNA so that an accurate measure of gene expression can be made. Currently, gene expression levels can be only qualitatively monitored in such fixed and embedded samples by using immunohistochemical staining to monitor protein expression levels.

The use of frozen tissue by health care professionals poses substantial inconveniences. Rapid biopsy delivery to avoid tissue and subsequent mRNA degradation is the primary concern when planning any RNA-based quantitative genetic marker assay. The health care professional performing the biopsy, must hastily deliver the tissue sample to a facility equipped to perform an RNA extraction protocol immediately upon tissue sample receipt. If no such facility is available, the clinician must promptly freeze the sample in order to prevent mRNA degradation. In order for the diagnostic facility to perform a useful RNA extraction protocol prior to tissue and RNA degradation, the tissue sample must remain frozen until it reaches the diagnostic facility, however far away that may be. Maintaining frozen tissue integrity during transport using specialized couriers equipped with liquid nitrogen and dry ice, comes only at a great expense.

Routine biopsies generally comprise a heterogenous mix of stromal and tumorous tissue. Unlike with fresh or frozen tissue, FPE biopsy tissue samples are readily microdissected and separated into stromal and tumor tissue and therefore, offer advantage over the use of fresh or frozen tissue. However, isolation of RNA
5 from fixed tissue, and especially fixed and paraffin embedded tissue, results in highly degraded RNA, which is generally not thought to be applicable to gene expression studies.

A number of techniques exist for the purification of RNA from biological samples, but none is reliable for isolation of RNA from FPE samples. For example,
10 Chomczynski (U.S. Pat. No. 5,346,994) describes a method for purifying RNA from tissues based on a liquid phase separation using phenol and guanidine isothiocyanate. A biological sample is homogenized in an aqueous solution of phenol and guanidine isothiocyanate and the homogenate thereafter mixed with chloroform. Following centrifugation, the homogenate separates into an organic
15 phase, an interphase and an aqueous phase. Proteins are sequestered in the organic phase, DNA in the interphase, and RNA in the aqueous phase. RNA can be precipitated from the aqueous phase. Unfortunately, this method is not applicable to fixed and paraffin-embedded (FPE) tissue samples.

Other known techniques for isolating RNA typically utilize either guanidine
20 salts or phenol extraction, as described for example in Sambrook, J. *et al.*, (1989) at pp. 7.3-7.24, and in Ausubel, F. M. *et al.*, (1994) at pp. 4.0.3-4.4.7. Again, none of the known methods provides reproducible quantitative results in the isolation of RNA from paraffin-embedded tissue samples.

Techniques for the isolation of RNA from paraffin-embedded tissues are thus particularly needed for the study of gene expression in tumor tissues, since expression levels of certain receptors or enzymes can then be used to determine the likelihood of success or appropriateness of a particular treatment.

- 5 We report here a significant association between high levels of the intratumoral *EGFR* mRNA and high levels of intratumoral *HER2-neu* mRNA with an inferior survivability. Accordingly, it is the object of the invention to provide a method of quantifying *EGFR* and/or *HER2-neu* mRNA from tumor tissue in order to provide an early prognosis for receptor tyrosine kinase targeted chemotherapies.
- 10 It is also the object of the invention to provide a method for assessing *EGFR* and/or *HER2-neu* levels in tissues fixed and paraffin-embedded (FPE) and predicting the probable sensitivity of a patient's tumor to treatment with receptor tyrosine kinase targeted chemotherapy by examining the amount *EGFR* and/or *HER2-neu* mRNA in a patient's tumor cells and comparing it to a predetermined threshold expression
- 15 level.

SUMMARY OF THE INVENTION

In one aspect of the invention there is provided a method for assessing levels of expression of *EGFR* mRNA obtained from fresh, frozen, fixed or fixed and paraffin-embedded (FPE) tumor cells.

5 In another aspect of the invention there is provided a method for assessing levels of expression of *HER2-neu* mRNA obtained from fresh, frozen, fixed or fixed and paraffin-embedded (FPE) tumor cells.

In another aspect of the invention there is provided a method of quantifying the amount of *EGFR* mRNA expression relative to an internal control from a fresh,
10 frozen, fixed or fixed and paraffin-embedded (FPE) tissue sample. This method includes isolation of total mRNA from said sample and determining the quantity of *EGFR* mRNA relative to the quantity of an internal control gene's mRNA.

In another aspect of the invention there is provided a method of quantifying the amount of *HER2-neu* mRNA expression relative to an internal control from a
15 fresh, frozen, fixed or fixed and paraffin-embedded (FPE) tissue sample. This method includes isolation of total mRNA from said sample and determining the quantity of *HER2-neu* mRNA relative to the quantity of an internal control gene's mRNA.

In an embodiment of this aspect of the invention, there are provided
20 oligonucleotide primers having the sequence of EGFR-1753F (SEQ ID NO: 1) or EGFR-1823R (SEQ ID NO:2) and sequences substantially identical thereto. The invention also provides for oligonucleotide primers having a sequence that hybridizes to SEQ ID NO: 1 or SEQ ID NO:2 or their complements under stringent conditions.

In another embodiment of this aspect of the invention, there are provided oligonucleotide primers having the sequence of HER2-neu 2671F (SEQ ID NO: 4) or HER2-neu 2699R (SEQ ID NO: 5) and sequences substantially identical thereto. The invention also provides for oligonucleotide primers having a sequence that
5 hybridizes to SEQ ID NO: 4 or SEQ ID NO: 5 or their complements under stringent conditions.

In yet another aspect of the invention there is provided a method for determining a receptor tyrosine kinase targeted chemotherapeutic regimen for a patient, comprising isolating RNA from a fresh, frozen, fixed or fixed and paraffin-
10 embedded (FPE) tumor sample; isolating RNA from a fresh, frozen, fixed or fixed and paraffin-embedded (FPE) matching non-malignant tissue sample; determining a gene expression level of *EGFR* in both samples; dividing the level of *EGFR* expression in the tumor sample with the *EGFR* expression level in the matching non-malignant tissue sample to determine a differential expression level; comparing
15 the differential *EGFR* gene expression level with a predetermined threshold level for the *EGFR* gene; and determining a chemotherapeutic regimen based on results of the comparison of the differential *EGFR* gene expression level with the predetermined threshold level.

In yet another aspect of the invention there is provided a method for
20 determining a receptor tyrosine kinase targeted chemotherapeutic regimen for a patient, comprising isolating RNA from a fresh, frozen, fixed or fixed and paraffin-embedded (FPE) tumor sample; isolating RNA from a fresh, frozen, fixed or fixed and paraffin-embedded (FPE) matching non-malignant tissue sample; determining a gene expression level of *HER2-neu* in both samples; dividing the level of *HER2-neu*

expression in the tumor sample with the *HER2-neu* expression level in the matching non-malignant tissue sample to determine a differential expression level; comparing the differential *HER2-neu* gene expression levels with a predetermined threshold level for the *HER2-neu* gene; and determining a chemotherapeutic regimen based on
5 results of the comparison of the differential *HER2-neu* gene expression level with the predetermined threshold level.

In yet another aspect of the invention there is provided a method for determining a receptor tyrosine kinase targeted chemotherapeutic regimen for a patient, comprising isolating RNA from a fresh, frozen, fixed or fixed and paraffin-
10 embedded (FPE) tumor sample; isolating RNA from a fresh, frozen, fixed or fixed and paraffin-embedded (FPE) matching non-malignant tissue sample; determining gene expression levels of *HER2-neu* and *EGFR* in both of the samples; dividing the level of *EGFR* expression in the tumor sample with the *EGFR* expression level in the matching non-malignant tissue sample to determine a *EGFR* differential
15 expression level; dividing the level of *HER2-neu* expression in the tumor sample with the *HER2-neu* expression level in the matching non-malignant tissue sample to determine a differential *HER2-neu* expression level; comparing the differential *HER2-neu* and *EGFR* gene expression levels with a predetermined threshold level for each of the *HER2-neu* and *EGFR* genes; and determining a chemotherapeutic
20 regimen based on results of the comparison of the differential *HER2-neu* and *EGFR* gene expression levels with the predetermined threshold levels.

In yet another aspect of the invention there is provided a method for determining the survivability of a patient, comprising isolating RNA from a fresh, frozen, fixed or fixed and paraffin-embedded (FPE) tumor sample; isolating RNA

from a fresh, frozen, fixed or fixed and paraffin-embedded (FPE) matching non-malignant tissue sample; determining a gene expression level of *EGFR* in both samples; dividing the level of *EGFR* expression in the tumor sample with the *EGFR* expression level in the matching non-malignant tissue sample to determine a differential expression level; comparing the differential *EGFR* gene expression level with a predetermined threshold level for the *EGFR* gene; and determining the survivability of a patient based on results of the comparison of the differential *EGFR* gene expression levels with the

In yet another aspect of the invention there is provided a method for determining the survivability of a patient, comprising isolating RNA from a fresh, frozen, fixed or fixed and paraffin-embedded (FPE) tumor sample; isolating RNA from a fresh, frozen, fixed or fixed and paraffin-embedded (FPE) matching non-malignant tissue sample; determining a gene expression level of *HER2-neu* in both samples; dividing the level of *HER2-neu* expression in the tumor sample with the *EGFR* expression level in the matching non-malignant tissue sample to determine a differential expression level; comparing the differential *HER2-neu* gene expression levels with a predetermined threshold level for the *HER2-neu* gene; and determining the survivability of a patient based on results of the comparison of the differential *HER2-neu* gene expression level with the predetermined threshold level.

In yet another aspect of the invention there is provided a method for determining the survivability of a patient, comprising isolating RNA from a fresh, frozen, fixed or fixed and paraffin-embedded (FPE) tumor sample; isolating RNA from a fresh, frozen, fixed or fixed and paraffin-embedded (FPE) matching non-

malignant tissue sample; determining gene expression levels of *HER2-neu* and *EGFR* in both of the samples; dividing the level of *EGFR* expression in the tumor sample with the *EGFR* expression level in the matching non-malignant tissue sample to determine a *EGFR* differential expression level; dividing the level of

5 *HER2-neu* expression in the tumor sample with the *HER2-neu* expression level in the matching non-malignant tissue sample to determine a *HER2-neu* differential expression level; comparing the differential *HER2-neu* and *EGFR* gene expression levels with a predetermined threshold level for each of the *HER2-neu* and *EGFR* genes; and determining the survivability of a patient based on results of the

10 comparison of the *EGFR* and *HER2-neu* gene expression levels with the predetermined threshold levels.

The invention further relates to a method of normalizing the uncorrected gene expression (UGE) of *EGFR* and *HER2-neu* relative to an internal control gene in a tissue sample analyzed using TaqMan® technology to known *EGFR* and *HER2-*

15 *neu* expression levels relative to an internal control from samples analyzed by pre-TaqMan® technology.

DESCRIPTION OF THE DRAWINGS

Figure 1. Estimated probability of survival of curatively resected non-small cell lung cancer patients versus the *HER2-neu* mRNA expression status. The median

20 survival was not reached in the low *HER2-neu* expression group compared to 31.1 months (95% C.I: 21.96- 40.24) in the high *HER2-neu* expression group($P=0.004$).

Figure 2. Estimated probability of survival of curatively resected non-small cell lung cancer patients versus the *EGFR* mRNA expression status. A trend towards

inferior overall survival was observable for the high *EGFR* expression group, but did not reach statistical significance. The median survival was not reached in the low *EGFR* expression group compared to 32.37 months (95% C.I: 8.43-56.31) in the high *EGFR* expressor group ($P=0.176$).

5 Figure 3. Estimated probability of survival of curatively resected non-small cell lung cancer patients versus combined patterns of *EGFR* and *HER2-neu* co-expression in NSCLC. The median survival showed low *HER2-neu* and *EGFR* expression, compared to 45.47 months in the high *EGFR* expression group, 31.10 months (95% C.I.: 14.77-47.43) in the high *HER2-neu* expression group, and 22.03 months (95% C.I: 2.30-41.76; $P=0.003$) in 10 the high *HER2-neu* and *EGFR* expression group.

Figure 4. Table showing high and low *EGFR* and *HER2-neu* expression in patients and tumors.

Figure 5. Table showing the survival of patients based on clinical and 15 molecular parameters.

Figure 6. Table showing Cox-proportional hazards regression models. Double marker refers to both *EGFR* and *HER2-neu* expression.

Figure 7 is a chart illustrating how to calculate *EGFR* expression relative to an internal control gene. The chart contains data obtained with two test samples, 20 (unknowns 1 and 2), and illustrates how to determine the uncorrected gene expression data (UGE). The chart also illustrates how to normalize UGE generated by the TaqMan® instrument with known relative *EGFR* values determined by pre-TaqMan® technology. This is accomplished by multiplying UGE to a correction

factor K_{EGFR} . The internal control gene in the figure is β -actin and the calibrator RNA is Human Liver Total RNA (Stratagene, Cat #735017).

Figure 8 is a chart illustrating how to calculate *HER2-neu* expression relative to an internal control gene. The chart contains data obtained with two test samples, (unknowns 1 and 2), and illustrates how to determine the uncorrected gene expression data (UGE). The chart also illustrates how to normalize UGE generated by the TaqMan® instrument with previously published *HER2-neu* values. This is accomplished by multiplying UGE to a correction factor $K_{HER2-neu}$. The internal control gene in the figure is β -actin and the calibrator RNA is Human Liver Total RNA (Stratagene, Cat #735017).

Figure 9 is a graph showing the corrected EGFR expression values of 5 different colon cancer patients' tumors. The patients were on a CPT-11/C225 receptor tyrosine kinase targeted treatment regimen. Patient 1 was determined to have a corrected EGFR expression level of 2.08×10^{-3} and had a completed response (CR). Patient 2 had a corrected EGFR expression level of 8.04×10^{-3} and had a partial response (PR). Patient 3 had a corrected EGFR expression level of 1.47×10^{-3} and also showed a partial response (PR). Patient 4 had a corrected EGFR expression level of 0.16×10^{-3} and had stable disease (SD) showing no response. Patient 5 had a no EGFR expression (0.0×10^{-3}) and had progressive disease (PR).

DETAILED DESCRIPTION OF THE INVENTION

Tumors expressing high levels of *HER2-neu* and/or *EGFR* mRNA are considered likely to be sensitive to receptor tyrosine kinase targeted chemotherapy. Conversely, those tumors expressing low amounts of *HER2-neu* and *EGFR* mRNA are not likely to be sensitive to receptor tyrosine kinase targeted chemotherapy. A patient's differential *HER2-neu* and *EGFR* mRNA expression status is judged by comparing it to a predetermined threshold expression level.

The invention provides a method of quantifying the amount of *HER2-neu* and/or *EGFR* mRNA expression in fresh, frozen, fixed or fixed and paraffin-embedded (FPE) tissue relative to gene expression of an internal control. The present inventors have developed oligonucleotide primers that allow accurate assessment of *HER2-neu* and *EGFR* gene expression in fresh, frozen, fixed or fixed and embedded tissues. The oligonucleotide primers, EGFR-1753F (SEQ ID NO: 1), EGFR-1823R (SEQ ID NO: 2), or oligonucleotide primers substantially identical thereto, preferably are used together with RNA extracted from fresh, frozen, fixed or fixed and paraffin embedded (FPE) tumor samples. The invention also provides oligonucleotide primers, *HER2-neu* 2671F (SEQ ID NO: 4), *HER2-neu* 2699R (SEQ ID NO: 5), or oligonucleotide primers substantially identical thereto, preferably are used together with RNA extracted from fresh, frozen, fixed or fixed and paraffin embedded (FPE) tumor samples. This measurement of *HER2-neu* and/or *EGFR* gene expression may then be used for prognosis of receptor tyrosine kinase targeted chemotherapy

This embodiment of the invention involves, a method for reliable extraction of RNA from fresh, frozen, fixed or FPE samples, determination of the content of

EGFR mRNA in the sample by using a pair of oligonucleotide primers, preferably oligonucleotide primer pair EGFR-1753F (SEQ ID NO: 1) and EGFR-1823R (SEQ ID NO: 2), or oligonucleotides substantially identical thereto, for carrying out reverse transcriptase polymerase chain reaction.

5 Another embodiment of the invention involves a method for reliable extraction of RNA from fresh, frozen, fixed or FPE samples, and determination of the content of *HER2-neu* mRNA in the sample by using a pair of oligonucleotide primers oligonucleotide primers, HER2-neu 2671F (SEQ ID NO: 4), HER2-neu 2699R (SEQ ID NO: 5), or oligonucleotide primers substantially identical thereto.

10 "Substantially identical" in the nucleic acid context as used herein, means hybridization to a target under stringent conditions, and also that the nucleic acid segments, or their complementary strands, when compared, are the same when properly aligned, with the appropriate nucleotide insertions and deletions, in at least about 60% of the nucleotides, typically, at least about 70%, more typically, at least
15 about 80%, usually, at least about 90%, and more usually, at least, about 95-98% of the nucleotides. Selective hybridization exists when the hybridization is more selective than total lack of specificity. See, Kanehisa, Nucleic Acids Res., 12:203-213 (1984).

 The methods of the present invention can be applied over a wide range of
20 tumor types. This allows for the preparation of individual "tumor expression profiles" whereby expression levels of *HER2-neu* and/or *EGFR* are determined in individual patient samples and response to various chemotherapeutics is predicted. Preferably, the methods of the invention are applied to solid tumors, most preferably NSCLC tumors.

A "differential expression level" as defined herein refers to the difference in the level of expression of either *EGFR* or *HER2-neu* in a tumor with respect to the level of expression of either *EGFR* or *HER2-neu* in a matching non-malignant tissue sample, respectively. The differential expression level is determined by dividing the UGE of a particular gene from the tumor sample with the UGE of the same gene from a matching non-malignant tissue sample.

A "predetermined threshold level", as defined herein relating to *EGFR* expression, is a level of differential *EGFR* expression above which (i.e., high), tumors are likely to be sensitive to a receptor tyrosine kinase targeted chemotherapeutic regimen. A high differential *EGFR* expression level is prognostic of lower patient survivability. Tumors with expression levels below this threshold level are not likely to be affected by a receptor tyrosine kinase targeted chemotherapeutic regimen. A low differential *EGFR* expression level is prognostic of higher patient survivability. Whether or not differential expression is above or below a "predetermined threshold level" is determined by the method used by Mafune et al., who calculated individual differential tumor/normal (T/N) expression ratios in matching non-malignant tissues obtained from patients with squamous cell carcinoma of the esophagus. Mafune et al., Clin Cancer Res 5:4073-4078, 1999. This method of analysis leads to a precise expression value for each patient, being based on the individual background expression obtained from matching non-malignant tissue. The differential expression of *EGFR* is considered "high" and indicative of low survivability if the UGE of *EGFR* : β -actin in a tumor sample divided by the UGE of *EGFR* : β -actin in a matching non-malignant tissue sample,

is above the predetermined threshold value of about 1.8. The differential expression of *EGFR* is considered "low" and indicative of high survivability if the UGE of *EGFR* : β -actin in a tumor sample divided by the UGE of *EGFR* : β -actin in a matching non-malignant tissue sample, is below the predetermined threshold value of about 1.8.

A "predetermined threshold level", as defined herein relating to differential *HER2-neu* expression, is a level of *HER2-neu* expression above which (i.e., high), tumors are likely to be sensitive to a receptor tyrosine kinase targeted chemotherapeutic regimen. A high differential *HER2-neu* expression level is prognostic of lower patient survivability. Tumors with expression levels below this threshold level are not likely to be affected by a receptor tyrosine kinase targeted chemotherapeutic regimen. A low differential *HER2-neu* expression level is prognostic of higher patient survivability. The differential expression of *HER2-neu* is considered "high" and indicative of low survivability if the UGE of *HER2-neu* : β -actin in a tumor sample divided by the UGE of *HER2-neu* : β -actin in a matching non-malignant tissue sample, is above the predetermined threshold value of about 1.8. The differential expression of *HER2-neu* is considered "low" and indicative of high survivability if the UGE of *HER2-neu* : β -actin in a tumor sample divided by the UGE of *HER2-neu* : β -actin in a matching non-malignant tissue sample, is below the predetermined threshold value of about 1.8.

A "threshold level" for *HER2-neu* was determined using the following results and method. The corrected *HER2-neu* mRNA expression, expressed as the ratio between *HER2-neu* and β -Actin PCR product, was 4.17×10^{-3} (range 0.28-

23.86 $\times 10^{-3}$) in normal lung and 4.35 $\times 10^{-3}$ (range: 0.21-68.11 $\times 10^{-3}$) in tumor tissue ($P=0.019$ Wilcoxon test). The maximal chi-square method by Miller and Siegmund (Miller et al., Biometrics 38:1011-1016, 1982) and Halpern (Biometrics 38:1017-1023, 1982) determined a threshold value of 1.8 to segregate patients into
5 low and high differential *HER2-neu* expressors. By this criterion, 29 (34.9%) patients had a high differential *HER2-neu* expression and 54 (65.1 %) had a low differential *HER2-neu* expression.

A "threshold level" for *EGFR* was determined using the following results and method. The median corrected *EGFR* mRNA expression was 8.17 $\times 10^{-3}$ (range:
10 0.31-46.26 $\times 10^{-3}$) in normal lung and 7.22 $\times 10^{-3}$ (range: 0.27-97.49 $\times 10^{-3}$) in tumor tissue ($P=n.s.$). The maximal chi-square method (Miller (1982); Halpern (1982)) determined a threshold value of 1.8 to segregate patients into low and high differential *EGFR* expressors. By this criterion, 28 (33.7%) patients had a high differential *EGFR* expression and 55 (66.3%) had a low differential *EGFR*
15 expression status.

In performing the method of the present invention either differential *EGFR* expression levels or differential *HER2-neu* expression levels are assayed in a patient to prognosticate the efficacy of a receptor tyrosine kinase targeted chemotherapeutic regimen. Moreover, in the method of the present invention differential *HER2-neu*
20 expression levels are assayed in a patient prognosticate the efficacy of a receptor tyrosine kinase targeted chemotherapeutic regimen. Additionally, in the method of the present invention differential *EGFR* expression levels are assayed in a patient to prognosticate the efficacy of a receptor tyrosine kinase targeted chemotherapeutic regimen. Alternatively, both differential *EGFR* expression levels and differential

HER2-neu expression levels are assayed in a patient to prognosticate the efficacy of a receptor tyrosine kinase targeted chemotherapeutic regimen.

"Matching non-malignant sample" as defined herein refers to a sample of non-cancerous tissue derived from the same individual as the tumor sample to be
5 analyzed for differential *EGFR* and/or differential *HER2-neu* expression. Preferably a matching non-malignant sample is derived from the same organ as the organ from which the tumor sample is derived. Most preferably, the matching non-malignant tumor sample is derived from the same organ tissue layer from which the tumor sample is derived. Also, it is preferable to take a matching non-malignant tissue
10 sample at the same time a tumor sample is biopsied. In a preferred embodiment tissues from the following two locations are analyzed: lung tumor and non-malignant lung tissue taken from the greatest distance form the tumor or colon tumor and non-malignant colon tissue taken from the greatest distance form the tumor as possible under the circumstances.

15 In performing the method of this embodiment of the present invention, tumor cells are preferably isolated from the patient. Solid or lymphoid tumors or portions thereof are surgically resected from the patient or obtained by routine biopsy. RNA isolated from frozen or fresh tumor samples is extracted from the cells by any of the methods typical in the art, for example, Sambrook, Fischer and
20 Maniatis, Molecular Cloning, a laboratory manual, (2nd ed.), Cold Spring Harbor Laboratory Press, New York, (1989). Preferably, care is taken to avoid degradation of the RNA during the extraction process.

However, tissue obtained from the patient after biopsy is often fixed, usually by formalin (formaldehyde) or gluteraldehyde, for example, or by alcohol

immersion. Fixed biological samples are often dehydrated and embedded in paraffin or other solid supports known to those of skill in the art. See Plenat *et al.*, Ann Pathol 2001 Jan;21(1):29-47. Non-embedded, fixed tissue as well as fixed and embedded tissue may also be used in the present methods. Solid supports for
5 embedding fixed tissue are envisioned to be removable with organic solvents for example, allowing for subsequent rehydration of preserved tissue.

RNA is extracted from paraffin-embedded (FPE) tissue cells by any of the methods as described in US Patent Application No. 09/469,338, filed December 20, 1999, which is hereby incorporated by reference in its entirety. As used herein, FPE
10 tissue means tissue that has been fixed and embedded in a solid removable support, such as storable or archival tissue samples. RNA may be isolated from an archival pathological sample or biopsy sample which is first deparaffinized. An exemplary deparaffinization method involves washing the paraffinized sample with an organic solvent, such as xylene, for example. Deparaffinized samples can be rehydrated
15 with an aqueous solution of a lower alcohol. Suitable lower alcohols, for example include, methanol, ethanol, propanols, and butanols. Deparaffinized samples may be rehydrated with successive washes with lower alcoholic solutions of decreasing concentration, for example. Alternatively, the sample is simultaneously deparaffinized and rehydrated. RNA is then extracted from the sample.

20 For RNA extraction, the fixed or fixed and deparaffinized samples can be homogenized using mechanical, sonic or other means of homogenization. Rehydrated samples may be homogenized in a solution comprising a chaotropic agent, such as guanidinium thiocyanate (also sold as guanidinium isothiocyanate). Homogenized samples are heated to a temperature in the range of about 50 to about

100 °C in a chaotropic solution, which contains an effective amount of a chaotropic agent, such as a guanidinium compound. A preferred chaotropic agent is guanidinium thiocyanate.

An "effective concentration of chaotropic agent" is chosen such that RNA is
5 purified from a paraffin-embedded sample in an amount of greater than about 10-fold that isolated in the absence of a chaotropic agent. Chaotropic agents include, for example: guanidinium compounds, urea, formamide, potassium iodide, potassium thiocyanate and similar compounds. The preferred chaotropic agent for the methods of the invention is a guanidinium compound, such as guanidinium
10 isothiocyanate (also sold as guanidinium thiocyanate) and guanidinium hydrochloride. Many anionic counterions are useful, and one of skill in the art can prepare many guanidinium salts with such appropriate anions. The effective concentration of guanidinium solution used in the invention generally has a concentration in the range of about 1 to about 5M with a preferred value of about
15 4M. If RNA is already in solution, the guanidinium solution may be of higher concentration such that the final concentration achieved in the sample is in the range of about 1 to about 5M. The guanidinium solution also is preferably buffered to a pH of about 3 to about 6, more preferably about 4, with a suitable biochemical buffer such as Tris-Cl. The chaotropic solution may also contain reducing agents,
20 such as dithiothreitol (DTT) and β -mercaptoethanol (BME). The chaotropic solution may also contain RNase inhibitors.

RNA is then recovered from the chaotropic solution by, for example, phenol chloroform extraction, ion exchange chromatography or size-exclusion chromatography. RNA may then be further purified using the techniques of

extraction, electrophoresis, chromatography, precipitation or other suitable techniques.

The quantification of *HER2-neu* or *EGFR* mRNA from purified total mRNA from fresh, frozen or fixed is preferably carried out using reverse-transcriptase polymerase chain reaction (RT-PCR) methods common in the art, for example. Other methods of quantifying of *HER2-neu* or *EGFR* mRNA include for example, the use of molecular beacons and other labeled probes useful in multiplex PCR. Additionally, the present invention envisages the quantification of *HER2-neu* and/or *EGFR* mRNA via use of a PCR-free systems employing, for example fluorescent labeled probes similar to those of the Invader® Assay (Third Wave Technologies, Inc.). Most preferably, quantification of *HER2-neu* and/or *EGFR* cDNA and an internal control or house keeping gene (e.g. β -actin) is done using a fluorescence based real-time detection method (ABI PRISM 7700 or 7900 Sequence Detection System [TaqMan®], Applied Biosystems, Foster City, CA.) or similar system as described by Heid *et al.*, (Genome Res 1996;6:986-994) and Gibson *et al.* (Genome Res 1996;6:995-1001). The output of the ABI 7700 (TaqMan® Instrument) is expressed in Ct's or "cycle thresholds". With the TaqMan® system, a highly expressed gene having a higher number of target molecules in a sample generates a signal with fewer PCR cycles (lower Ct) than a gene of lower relative expression with fewer target molecules (higher Ct).

As used herein, a "house keeping" gene or "internal control" is any constitutively or globally expressed gene whose presence enables an assessment of *HER2-neu* and/or *EGFR* mRNA levels. Such an assessment comprises a

determination of the overall constitutive level of gene transcription and a control for variations in RNA recovery. "House-keeping" genes or "internal controls" can include, but are not limited to the cyclophilin gene, β -actin gene, the transferrin receptor gene, GAPDH gene, and the like. Most preferably, the internal control
5 gene is β -actin gene as described by Eads *et al.*, Cancer Research 1999; 59:2302-2306.

A control for variations in RNA recovery requires the use of "calibrator RNA." The "calibrator RNA" is intended to be any available source of accurately pre-quantified control RNA. Preferably, Human Liver Total RNA (Stratagene, Cat
10 #735017) is used.

"Uncorrected Gene Expression (UGE)" as used herein refers to the numeric output of *HER2-neu* and/or *EGFR* expression relative to an internal control gene generated by the TaqMan® instrument. The equation used to determine UGE is shown in Examples 3 and 4, and illustrated with sample calculations in Figures 7
15 and 8.

These numerical values allow the determination of whether or not the differential gene expression (i.e., "UGE" or of a particular tumor sample divided by the "UGE" of a matching non-tumor sample) falls above or below the "predetermined threshold" level. The predetermined threshold level for *EGFR* and
20 *HER2-neu* is about 1.8.

A further aspect of this invention provides a method to normalize uncorrected gene expression (UGE) values acquired from the TaqMan® instrument with "known relative gene expression" values derived from non-TaqMan®

technology. Preferably, TaqMan® derived *HER2-neu* and/or *EGFR* UGE values from a tissue sample are normalized to samples with known non-TaqMan® derived relative *HER2-neu* and/or *EGFR* : β -actin expression values.

"Corrected Relative *EGFR* Expression" as used herein refers to normalized
5 *EGFR* expression whereby UGE is multiplied with a *EGFR* specific correction factor (K_{EGFR}), resulting in a value that can be compared to a known range of *EGFR* expression levels relative to an internal control gene. Example 3 and Figure 7 illustrate these calculations in detail. K_{EGFR} specific for *EGFR*, the internal control β -actin and calibrator Human Liver Total RNA (Stratagene, Cat #735017), is 26.95
10 $\times 10^{-3}$. These numerical values also allow the determination of whether or not the "Corrected Relative Expression" of a particular tumor sample divided by the "Corrected Relative Expression" of a matching non-tumor sample (i.e., differential expression) falls above or below the "predetermined threshold" level. The predetermined threshold level for *HER2-neu* or *EGFR* is about 1.8. In determining
15 whether the differential expression of either *EGFR* or *HER2-neu* in a tumor sample is 1.8 times greater than in a matching non-tumor sample, one will readily recognize that either UGE values or Corrected Relative Expression values can be used. For example, if one divides the Corrected Relative Expression level of the tumor with that of the matching non-tumor sample, the K-factor cancels out and one is left with
20 same ratio as if one had used UGE values.

"Known relative gene expression" values are derived from previously analyzed tissue samples and are based on the ratio of the RT-PCR signal of a target gene to a constitutively expressed internal control gene (e.g. β -Actin, GAPDH, etc.).

Preferably such tissue samples are formalin fixed and paraffin-embedded (FPE) samples and RNA is extracted from them according to the protocol described in Example 1. To quantify gene expression relative to an internal control standard quantitative RT-PCR technology known in the art is used. Pre-TaqMan®

5 technology PCR reactions are run for a fixed number of cycles (i.e., 30) and endpoint values are reported for each sample. These values are then reported as a ratio of *EGFR* expression to β -actin expression.

K_{EGFR} may be determined for an internal control gene other than β -actin and/or a calibrator RNA different than Human Liver Total RNA (Stratagene, Cat
10 #735017). To do so, one must calibrate both the internal control gene and the calibrator RNA to tissue samples for which *EGFR* expression levels relative to that particular internal control gene have already been determined (i.e., "known relative gene expression"). Preferably such tissue samples are formalin fixed and paraffin-embedded (FPE) samples and RNA is extracted from them according to the protocol
15 described in Example 1. Such a determination can be made using standard pre-TaqMan®, quantitative RT-PCR techniques well known in the art. Upon such a determination, such samples have "known relative gene expression" levels of *EGFR* useful in the determining a new K_{EGFR} specific for the new internal control and/or calibrator RNA as described in Example 3.

20 "Corrected Relative *HER2-neu* Expression" as used herein refers to normalized *HER2-neu* expression whereby UGE is multiplied with a *HER2-neu* specific correction factor ($K_{HER2-neu}$), resulting in a value that can be compared to a known range of *HER2-neu* expression levels relative to an internal control gene.

Example 4 and Figure 8 illustrate these calculations in detail. $K_{HER2-neu}$ specific for *HER2-neu*, the internal control β -actin and calibrator Human Liver Total RNA (Stratagene, Cat #735017), is 13.3×10^{-3} .

$K_{HER2-neu}$ may be determined for an internal control gene other than β -actin
5 and/or a calibrator RNA different than Human Liver Total RNA (Stratagene, Cat #735017). To do so, one must calibrate both the internal control gene and the calibrator RNA to tissue samples for which *HER2-neu* expression levels relative to that particular internal control gene have already been determined (i.e., "known relative gene expression"). Preferably such tissue samples are formalin fixed and
10 paraffin-embedded (FPE) samples and RNA is extracted from them according to the protocol described in herein. Such a determination can be made using standard pre-TaqMan®, quantitative RT-PCR techniques well known in the art, for example. Upon such a determination, such samples have "known relative gene expression" levels of *HER2-neu* useful in the determining a new $K_{HER2-neu}$ specific for the new
15 internal control and/or calibrator RNA as described in Example 4.

The methods of the invention are applicable to a wide range of tissue and tumor types and so can be used for assessment of clinical treatment of a patient and as a diagnostic or prognostic tool for a range of cancers including breast, head and neck, lung, esophageal, colorectal, and others. In a preferred embodiment, the
20 present methods are applied to prognosis of NSCLC tumors.

Pre-chemotherapy treatment tumor biopsies are usually available only as fixed paraffin embedded (FPE) tissues, generally containing only a very small amount of heterogeneous tissue. Such FPE samples are readily amenable to

microdissection, so that *HER2-neu* and/or *EGFR* gene expression may be determined in tumor tissue uncontaminated with non-malignant stromal tissue. Additionally, comparisons can be made between non-malignant stromal and tumor tissue within a biopsy tissue sample, since such samples often contain both types of
5 tissues.

Generally, any oligonucleotide pairs that flank a region of *EGFR* gene, as shown in SEQ ID NO: 10, may be used to carry out the methods of the invention. Primers hybridizing under stringent conditions to a region of the *EGFR* gene for use in the present invention will amplify a product between 20-1000 base pairs,
10 preferably 50-100 base pairs, most preferably less than 100 base pairs.

The invention provides specific oligonucleotide primer pairs and oligonucleotide primers substantially identical thereto, that allow particularly accurate assessment of *EGFR* expression using fresh, frozen, fixed or FPE tissues. Preferable are oligonucleotide primers, EGFR-1753F (SEQ ID NO: 1) and EGFR-
15 1823R (SEQ ID NO: 2), (also referred to herein as the oligonucleotide primer pair EGFR) and oligonucleotide primers substantially identical thereto. The oligonucleotide primers EGFR-1753F (SEQ ID NO: 1) and EGFR-1823R, (SEQ ID NO: 2) have been shown to be particularly effective for measuring *EGFR* mRNA levels using RNA extracted from fresh, frozen, fixed or FPE cells by any of the
20 methods for mRNA isolation, for example as described Example 1.

Furthermore, any oligonucleotide pairs that flank a region of *HER2-neu* gene, as shown in SEQ ID NO: 11, may be used to carry out the methods of the invention. Primers hybridizing under stringent conditions to a region of the *HER2-neu* gene for use in the present invention will amplify a product between about 20-

1000 base pairs, preferably about 50-100 base pairs, most preferably less than about 100 base pairs.

The invention provides specific oligonucleotide primers pairs and oligonucleotide primers substantially identical thereto, that allow particularly
5 accurate assessment of *HER2-neu* expression in fresh, frozen, fixed or FPE tissues. Preferable are oligonucleotide primers, HER2-neu 2671F (SEQ ID NO: 4) and HER2-neu 2699R (SEQ ID NO: 5), (also referred to herein as the oligonucleotide primer pair HER2-neu) and oligonucleotide primers substantially identical thereto. The oligonucleotide primers HER2-neu 2671F (SEQ ID NO: 4) and HER2-neu
10 2699R (SEQ ID NO: 5) have been shown to be particularly effective for measuring *HER2-neu* mRNA levels using RNA extracted from fresh, frozen, fixed or FPE cells by any of the methods for mRNA isolation, for example as described herein.

This invention includes substantially identical oligonucleotides that hybridize under stringent conditions (as defined herein) to all or a portion of the
15 oligonucleotide primer sequence of EGFR-1753F (SEQ ID NO: 1), its complement or EGFR-1823R (SEQ ID NO: 2), or its complement or oligonucleotide primer sequence of HER2-neu 2671F (SEQ ID NO: 4), its complement or HER2-neu 2699R (SEQ ID NO: 5), or its complement.

Under stringent hybridization conditions, only highly complementary, i.e.,
20 substantially similar nucleic acid sequences as defined herein hybridize. Preferably, such conditions prevent hybridization of nucleic acids having 4 or more mismatches out of 20 contiguous nucleotides, more preferably 2 or more mismatches out of 20 contiguous nucleotides, most preferably one or more mismatch out of 20 contiguous nucleotides.

The hybridizing portion of the nucleic acids is typically at least about 10 (e.g., 15) nucleotides in length. The hybridizing portion of the hybridizing nucleic acid is at least about 80%, preferably at least about 95%, or most preferably about at least 98%, identical to the sequence of a portion or all of oligonucleotide primer

5 EGFR-1753F (SEQ ID NO: 1), its complement or EGFR-1823R (SEQ ID NO: 2), or its complement or oligonucleotide primer HER2-neu 2671F (SEQ ID NO: 4), its complement or HER2-neu 2699R (SEQ ID NO: 5), or its complement.

Hybridization of the oligonucleotide primer to a nucleic acid sample under stringent conditions is defined below. Nucleic acid duplex or hybrid stability is

10 expressed as a melting temperature (T_m), which is the temperature at which the probe dissociates from the target DNA. This melting temperature is used to define the required stringency conditions. If sequences are to be identified that are substantially identical to the probe, rather than identical, then it is useful to first establish the lowest temperature at which only homologous hybridization occurs

15 with a particular concentration of salt (e.g. SSC or SSPE). Then assuming that 1% mismatching results in a 1°C decrease in T_m , the temperature of the final wash in the hybridization reaction is reduced accordingly (for example, if sequences having >95% identity with the probe are sought, the final wash temperature is decreased by 5°C). In practice, the change in T_m can be between 0.5°C and 1.5°C per 1% mismatch.

20

Stringent conditions involve hybridizing at about 68°C in 5x SSC/5x Denhart's solution/1.0% SDS, and washing in 0.2x SSC/0.1% SDS at room temperature. Moderately stringent conditions include washing in 3x SSC at about 42°C. The parameters of salt concentration and temperature be varied to achieve

optimal level of identity between the primer and the target nucleic acid. Additional guidance regarding such conditions is readily available in the art, for example, Sambrook, Fischer and Maniatis, *Molecular Cloning, a laboratory manual*, (2nd ed.), Cold Spring Harbor Laboratory Press, New York, (1989) and F. M. Ausubel et al eds., *Current Protocols in Molecular Biology*, John Wiley and Sons (1994).

Oligonucleotide primers disclosed herein are capable of allowing accurate assessment of *HER2-neu* and/or *EGFR* gene expression in a fixed or fixed and paraffin embedded tissue, as well as frozen or fresh tissue. This is despite the fact that RNA derived from FPE samples is more fragmented relative to that of fresh or frozen tissue. Thus, the methods of the invention are suitable for use in assaying *HER2-neu* and/or *EGFR* gene expression levels in all tissues where previously there existed no accurate and consistent way to assay *HER2-neu* and/or *EGFR* gene in fresh and frozen tissues and no way at all to assay *HER2-neu* and/or *EGFR* gene expression using fixed tissues.

Over-activity of *HER2-neu* refers to either an amplification of the gene encoding *HER2-neu* or the production of a level of *HER2-neu* activity which can be correlated with a cell proliferative disorder (i.e., as the level of *HER2-neu* increases the severity of one or more of the symptoms of the cell proliferative disorder increases).

A "receptor tyrosine kinase targeted" chemotherapy or chemotherapeutic regimen in the context of the present invention refers a chemotherapy comprising agents that specifically interfere with Class I receptor tyrosine kinase function. Preferably, such agents will inhibit *EGFR* and/or *HER2-neu* receptor tyrosine kinase signaling activity. Such agents include 4-anilinoquinazolines such as

6-acrylamido-4-anilinoquinazoline Bonvini et al., Cancer Res. 2001 Feb
15;61(4):1671-7 and derivatives, erbstatin (Toi et al., Eur. J. Cancer Clin. Oncol.,
1990, 26, 722.), Geldanamycin, bis monocyclic, bicyclic or heterocyclic aryl
compounds (PCT WO 92/20642), vinylene-azaindole derivatives (PCT WO
5 94/14808) and 1-cyclopropyl-4-pyridyl-quinolones (U.S. Pat. No. 5,330,992)
which have been described generally as tyrosine kinase inhibitors. Also, Styryl
compounds (U.S. Pat. No. 5,217,999), styryl-substituted pyridyl compounds (U.S.
Pat. No. 5,302,606), certain quinazoline derivatives (EP Application No. 0 566 266
A1), seleoindoles and selenides (PCT WO 94/03427), tricyclic polyhydroxylic
10 compounds (PCT WO 92/21660) and benzylphosphonic acid compounds (PCT WO
91/15495) have been described as compounds for use as tyrosine kinase inhibitors
for use in the treatment of cancer.

Other agents targeting EGFR and/or HER2-neu receptor tyrosine kinase
signaling activity include antibodies that inhibit growth factor receptor biological
15 function indirectly by mediating cytotoxicity via a targeting function

Antibodies complexing with the receptor activates serum complement and/or
mediate antibody-dependent cellular cytotoxicity. The antibodies which bind the
receptor can also be conjugated to a toxin (immunotoxins). Advantageously
antibodies are selected which greatly inhibit the receptor function by binding the
20 steric vicinity of the ligand binding site of the receptor (blocking the receptor),
and/or which bind the growth factor in such a way as to prevent (block) the ligand
from binding to the receptor. These antibodies are selected using conventional in
vitro assays for selecting antibodies which neutralize receptor function. Antibodies
that act as ligand agonists by mimicking the ligand are discarded by conducting

suitable assays as will be apparent to those skilled in the art. For certain tumor cells, the antibodies inhibit an autocrine growth cycle (i.e. where a cell secretes a growth factor which then binds to a receptor of the same cell). Since some ligands, e.g. TGF- α , are found lodged in cell membranes, the antibodies serving a targeting
5 function are directed against the ligand and/or the receptor

The cytotoxic moiety of the immunotoxin may be a cytotoxic drug or an enzymatically active toxin of bacterial or plant origin, or an enzymatically active fragment of such a toxin. Enzymatically active toxins and fragments thereof used are diphtheria, nonbinding active fragments of diphtheria toxin, exotoxin (from
10 *Pseudomonas aeruginosa*), ricin, abrin, modeccin, alpha-sarcin, *Aleurites fordii* proteins, dianthin proteins, *Phytolacca americana* proteins (PAPI, PAPII, and PAP-S), *momordica charantia* inhibitor, curcin, crotin, *sapaonaria officinalis* inhibitor, gelonin, mitogellin, restrictocin, phenomycin, and enomycin. In another embodiment, the antibodies are conjugated to small molecule anticancer drugs.
15 Conjugates of the monoclonal antibody and such cytotoxic moieties are made using a variety of bifunctional protein coupling agents. Examples of such reagents are SPDP, IT, bifunctional derivatives of imidoesters such as dimethyl adipimidate HCl, active esters such as disuccinimidyl suberate, aldehydes such as glutaraldehyde, bis-azido compounds such as bis (p-azidobenzoyl) hexanediamine, bis-diazonium
20 derivatives such as bis-(p-diazoniumbenzoyl)-ethylenediamine, diisocyanates such as tolylene 2,6-diisocyanate, and bis-active fluorine compounds such as 1,5-difluoro-2,4-dinitrobenzene. The lysing portion of a toxin may be joined to the Fab fragment of the antibodies.

Cytotoxic radiopharmaceuticals for treating cancer may be made by conjugating radioactive isotopes to the antibodies. The term "cytotoxic moiety" as used herein is intended to include such isotopes.

In another embodiment, liposomes are filled with a cytotoxic drug and the liposomes are coated with antibodies specifically binding a growth factor receptor. Since there are many receptor sites, this method permits delivery of large amounts of drug to the appropriate cell type. The exact formulation, route of administration and dosage can be chosen by the individual physician in view of the patient's condition. (See e.g. Fingl et al., in *The Pharmacological Basis of Therapeutics*, 1975, Ch. 1 p. 1). It should be noted that the attending physician would know how and when to terminate, interrupt, or adjust administration due to toxicity, or organ dysfunctions. Conversely, the attending physician would also know to adjust treatment to higher levels if the clinical response were not adequate (precluding toxicity). The magnitude of an administered dose in the management of the oncogenic disorder of interest will vary with the severity of the condition to be treated and to the route of administration. The severity of the condition may, for example, be evaluated, in part, by standard prognostic evaluation methods. Further, the dose and perhaps dose frequency, will also vary according to the age, body weight, and response of the individual patient.

Depending on the specific conditions being treated, such agents may be formulated and administered systemically or locally. Techniques for formulation and administration may be found in *Remington's Pharmaceutical Sciences*, 18th ed., Mack Publishing Co., Easton, Pa. (1990). Suitable routes may include oral, rectal,

transdermal, vaginal, transmucosal, or intestinal administration; parenteral delivery, including intramuscular, subcutaneous, intramedullary injections, as well as intrathecal, direct intraventricular, intravenous, intraperitoneal, intranasal, or intraocular injections, just to name a few. For injection, the agents of the invention
5 may be formulated in aqueous solutions, preferably in physiologically compatible buffers such as Hanks's solution, Ringer's solution, or physiological saline buffer. For such transmucosal administration, penetrants appropriate to the barrier to be permeated are used in the formulation. Such penetrants are generally known in the art.

10 The invention being thus described, practice of the invention is illustrated by the experimental examples provided below. The skilled practitioner will realize that the materials and methods used in the illustrative examples can be modified in various ways. Such modifications are considered to fall within the scope of the present invention.

15

EXAMPLES

EXAMPLE 1

RNA Isolation from FPE Tissue

RNA is extracted from paraffin-embedded tissue by the following general
20 procedure.

A. Deparaffinization and hydration of sections:

(1) A portion of an approximately 10 μ M section is placed in a 1.5 mL plastic centrifuge tube.

(2) 600 μ L, of xylene are added and the mixture is shaken vigorously for about 10 minutes at room temperature (roughly 20 to 25 °C).

5 (3) The sample is centrifuged for about 7 minutes at room temperature at the maximum speed of the bench top centrifuge (about 10-20,000 x g).

(4) Steps 2 and 3 are repeated until the majority of paraffin has been dissolved. Two or more times are normally required depending on the amount of paraffin included in the original sample portion.

10 (5) The xylene solution is removed by vigorously shaking with a lower alcohol, preferably with 100% ethanol (about 600 μ L) for about 3 minutes.

(6) The tube is centrifuged for about 7 minutes as in step (3). The supernatant is decanted and discarded. The pellet becomes white.

(7) Steps 5 and 6 are repeated with successively more dilute ethanol
15 solutions: first with about 95% ethanol, then with about 80% and finally with about 70% ethanol.

(8) The sample is centrifuged for 7 minutes at room temperature as in step.

(9) The supernatant is discarded and the pellet is allowed to dry at room temperature for about 5 minutes.

20 **B. RNA Isolation with Phenol-Chloroform**

(1) 400 μ L guanidine isothiocyanate solution including 0.5% sarcosine and 8 μ L dithiothreitol is added.

(2) The sample is then homogenized with a tissue homogenizer (Ultra-Turrax, IKA-Works, Inc., Wilmington, NC) for about 2 to 3 minutes while gradually increasing the speed from low speed (speed 1) to high speed (speed 5).

(3) The sample is then heated at about 95 °C for about 5-20 minutes. It is preferable to pierce the cap of the tube containing the sample with a fine gauge needle before heating to 95 °C. Alternatively, the cap may be affixed with a plastic clamp or with laboratory film.

(4) The sample is then extracted with 50 µL 2M sodium acetate at pH 4.0 and 600 µL of phenol/chloroform/isoamyl alcohol (10:1.93:0.036), prepared fresh by mixing 18 mL phenol with 3.6 mL of a 1:49 isoamyl alcohol:chloroform solution. The solution is shaken vigorously for about 10 seconds then cooled on ice for about 15 minutes.

(5) The solution is centrifuged for about 7 minutes at maximum speed. The upper (aqueous) phase is transferred to a new tube.

(6) The RNA is precipitated with about 10 µL glycogen and with 400 µL isopropanol for 30 minutes at -20 °C.

(7) The RNA is pelleted by centrifugation for about 7 minutes in a benchtop centrifuge at maximum speed; the supernatant is decanted and discarded; and the pellet washed with approximately 500 µL of about 70 to 75% ethanol.

(8) The sample is centrifuged again for 7 minutes at maximum speed. The supernatant is decanted and the pellet air dried. The pellet is then dissolved in an appropriate buffer for further experiments (e.g., 50 µL 5mM Tris chloride, pH 8.0).

EXAMPLE 2

mRNA Reverse Transcription and PCR

Reverse Transcription: RNA was isolated from microdissected or non-microdissected formalin fixed paraffin embedded (FPE) tissue as illustrated in Example 1, or from fresh or frozen tissue by a single step guanidinium isocyanate method using the QuickPrep™ *Micro* mRNA purification kit (Amersham Pharmacia Biotech Inc., Piscataway, N.J.) according to the manufacturer's instructions. After precipitation with ethanol and centrifugation, the RNA pellet was dissolved in 50 ul of 5 mM Tris/Cl at pH 8.0. M-MLV Reverse Transcriptase will extend an oligonucleotide primer hybridized to a single-stranded RNA or DNA template in the presence of deoxynucleotides, producing a complementary strand. The resulting RNA was reverse transcribed with random hexamers and M-MLV Reverse Transcriptase from Life Technologies. The reverse transcription was accomplished by mixing 25 µl of the RNA solution with 25.5 µl of "reverse transcription mix" (see below). The reaction was placed in a thermocycler for 8 min at 26° C (for binding the random hexamers to RNA), 45 min at 42° C (for the M-MLV reverse transcription enzymatic reaction) and 5 min at 95° C (for heat inactivation of DNase).

"Reverse transcription mix" consists of 10 ul 5X buffer (250 mM Tris-HCl, pH 8.3, 375 mM KCl, 15 mM MgCl₂), 0.5 ul random hexamers (50 O.D. dissolved in 550 ul of 10 mM Tris-HCl pH 7.5) 5 ul 10 mM dNTPs (dATP, dGTP, dCTP and dTTP), 5 ul 0.1 M DTT, 1.25 ul BSA (3mg/ml in 10 mM Tris-HCL, pH 7.5), 1.25 ul RNA Guard 24,800U/ml (RNAse inhibitor) (Porcine #27-0816, Amersham Pharmacia) and 2.5 ul MMLV 200U/ul (Life Tech Cat #28025-02).

Final concentrations of reaction components are: 50 mM Tris-HCl, pH 8.3, 75 mM KCl, 3 mM MgCl₂, 1.0 mM dNTP, 1.0 mM DTT, 0.00375 mg/ml BSA, 0.62 U/ul RNA Guard and 10 U/ul MMLV.

PCR Quantification of mRNA expression. Quantification of *EGFR* cDNA

- 5 and an internal control or house keeping gene (e.g., β -actin) cDNA was done using a fluorescence based real-time detection method (ABI PRISM 7700 or 7900 Sequence Detection System [TaqMan®], Applied Biosystems, Foster City, CA.) as described by Heid *et al.*, (Genome Res 1996;6:986-994); Gibson *et al.*, (Genome Res 1996;6:995-1001). In brief, this method uses a dual labelled fluorogenic
- 10 TaqMan® oligonucleotide probe, (EGFR-1773 (SEQ ID NO: 3), $T_m = 70^\circ \text{C}$; HER2-neu 2657 (SEQ ID NO: 6), β -actin-611 (SEQ ID NO: 7) that anneals specifically within the forward and reverse primers. Laser stimulation within the capped wells containing the reaction mixture causes emission of a 3'quencher dye (TAMRA) until the probe is cleaved by the 5' to 3'nuclease activity of the DNA
- 15 polymerase during PCR extension, causing release of a 5' reporter dye (6FAM). Production of an amplicon thus causes emission of a fluorescent signal that is detected by the TaqMan®'s CCD (charge-coupled device) detection camera, and the amount of signal produced at a threshold cycle within the purely exponential phase of the PCR reaction reflects the starting copy number of the sequence of interest.
- 20 Comparison of the starting copy number of the sequence of interest with the starting copy number of the internal control gene provides a relative gene expression level. TaqMan® analyses yield levels that are expressed as ratios between two absolute measurements (gene of interest/internal control gene).

The PCR reaction mixture consisted 0.5 μ l of the reverse transcription reaction containing the cDNA prepared as described above 600 nM of each oligonucleotide primers EGFR-1753F (SEQ ID NO:1, T_m = 59 $^{\circ}$ C) and EGFR-1823R (SEQ ID NO: 2, T_m = 58 $^{\circ}$ C) or oligonucleotide primers HER2-neu 2671F (SEQ ID NO:4) and HER2-neu 2699R (SEQ ID NO: 5) 200 nM TaqMan $^{\circledR}$ probe (SEQ ID NO:3 or SEQ ID NO: 6), 5 U AmpliTaq Gold Polymerase, 200 μ M each dATP, dCTP, dGTP, 400 μ M dTTP, 5.5 mM MgCl₂, and 1 \times Taqman Buffer A containing a reference dye, to a final volume of less than or equal to 25 μ l (all reagents Applied Biosystems, Foster City, CA). Cycling conditions were, 95 $^{\circ}$ C for 10 min, followed by 45 cycles at 95 $^{\circ}$ C for 15s and 60 $^{\circ}$ C for 1 min. Oligonucleotides used to quantify internal control gene β -Actin were β -Actin-592F (SEQ ID NO: 8) and β -Actin-651R (SEQ ID NO: 9).

EXAMPLE 3

Determining the Uncorrected Gene Expression (UGE) for EGFR

Two pairs of parallel reactions are carried out. The "test" reactions and the "calibration" reactions. Figure 7. The *EGFR* amplification reaction and the β -actin internal control amplification reaction are the test reactions. Separate *EGFR* and β -actin amplification reactions are performed on the calibrator RNA template and are referred to as the calibration reactions. The TaqMan $^{\circledR}$ instrument will yield four different cycle threshold (Ct) values: Ct_{EGFR} and Ct _{β -actin} from the test reactions and Ct_{EGFR} and Ct _{β -actin} from the calibration reactions. The differences in Ct values for the two reactions are determined according to the following equation:

$$\begin{aligned}\Delta Ct_{\text{test}} &= Ct_{EGFR} - Ct_{\beta\text{-actin}} && \text{(From the "test" reaction)} \\ \Delta Ct_{\text{calibrator}} &= Ct_{EGFR} - Ct_{\beta\text{-actin}} && \text{(From the "calibration" reaction)}\end{aligned}$$

Next the step involves raising the number 2 to the negative ΔCt , according to
 5 the following equations.

$$\begin{aligned}2^{-\Delta Ct_{\text{test}}} &&& \text{(From the "test" reaction)} \\ 2^{-\Delta Ct_{\text{calibrator}}} &&& \text{(From the "calibration" reaction)}\end{aligned}$$

In order to then obtain an uncorrected gene expression for *EGFR* from the
 TaqMan® instrument the following calculation is carried out:

10

$$\text{Uncorrected gene expression (UGE) for } EGFR = 2^{-\Delta Ct_{\text{test}}} / 2^{-\Delta Ct_{\text{calibrator}}}$$

Normalizing UGE with known relative EGFR expression levels

The normalization calculation entails a multiplication of the UGE with a
 15 correction factor (K_{EGFR}) specific to *EGFR* and a particular calibrator RNA. A
 correction factor K_{EGFR} can also be determined for any internal control gene and any
 accurately pre-quantified calibrator RNA. Preferably, the internal control gene β -
 actin and the accurately pre-quantified calibrator RNA, Human Liver Total RNA
 (Stratagene, Cat #735017), are used. Given these reagents correction factor K_{EGFR}
 20 equals 1.54.

Normalization is accomplished using a modification of the ΔCt method
 described by Applied Biosystems, the TaqMan® manufacturer, in User Bulletin #2
 and described above. To carry out this procedure, the UGE of 6 different FPE test
 tissues were analyzed for *EGFR* expression using the TaqMan® methodology

described above. The internal control gene β -actin and the calibrator RNA, Human Liver Total RNA (Stratagene, Cat #735017) was used.

- The already known relative *EGFR* expression level of each sample AG221, AG222, AG252, Adult Lung, PC3, AdCol was divided by its corresponding
- 5 TaqMan® derived UGE to yield an unaveraged correction factor K.

$$K_{\text{unaveraged}} = \text{Known Values} / \text{UGE}$$

- Next, all of the K values are averaged to determine a single K_{EGFR} correction factor specific for *EGFR*, Stratgene Human Liver Total RNA (Stratagene, Cat
- 10 #735017) from calibrator RNA and β -actin.

- Therefore, to determine the Corrected Relative *EGFR* Expression in an unknown tissue sample on a scale that is consistent with pre-TaqMan® *EGFR* expression studies, one merely multiplies the uncorrected gene expression data (UGE) derived from the TaqMan® apparatus with the K_{EGFR} specific correction
- 15 factor, given the use of the same internal control gene and calibrator RNA.

$$\text{Corrected Relative } EGFR \text{ Expression} = \text{UGE} \times K_{EGFR}$$

- A K_{EGFR} may be determined using any accurately pre-quantified calibrator RNA or internal control gene. Future sources of accurately pre-quantified RNA can
- 20 be calibrated to samples with known relative *EGFR* expression levels as described in the method above or may now be calibrated against a previously calibrated calibrator RNA such as Human Liver Total RNA (Stratagene, Cat #735017) described above.

For example, if a subsequent K_{EGFR} is determined for a different internal control gene and/or a different calibrator RNA, one must calibrate both the internal control gene and the calibrator RNA to tissue samples for which *EGFR* expression levels relative to that particular internal control gene have already been determined.

- 5 Such a determination can be made using standard pre-TaqMan®, quantitative RT-PCR techniques well known in the art. The known expression levels for these samples will be divided by their corresponding UGE levels to determine a K for that sample. K values are then averaged depending on the number of known samples to determine a new K_{EGFR} specific to the different internal control gene and/or
- 10 calibrator RNA.

EXAMPLE 4

Determining the Uncorrected Gene Expression (UGE) for HER2-neu

- Two pairs of parallel reactions are carried out. The "test" reactions and the "calibration" reactions. Figure 8. The *HER2-neu* amplification reaction and the β -
- 15 actin internal control amplification reaction are the test reactions. Separate *HER2-neu* and β -actin amplification reactions are performed on the calibrator RNA template and are referred to as the calibration reactions. The TaqMan® instrument will yield four different cycle threshold (Ct) values: $Ct_{HER2-neu}$ and $Ct_{\beta-actin}$ from the test reactions and $Ct_{Her2-neu}$ and $Ct_{\beta-actin}$ from the calibration reactions. The differences
- 20 in Ct values for the two reactions are determined according to the following equation:

$$\Delta Ct_{test} = Ct_{HER2-neu} - Ct_{\beta-actin}$$

(From the "test" reaction)

25 $\Delta Ct_{calibrator} = Ct_{Her2-neu} - Ct_{\beta-actin}$

(From the "calibration" reaction)

Next the step involves raising the number 2 to the negative ΔC_t , according to the following equations.

$$\begin{array}{ll} 2^{-\Delta C_t_{\text{test}}} & \text{(From the "test" reaction)} \\ 2^{-\Delta C_t_{\text{calibrator}}} & \text{(From the "calibration" reaction)} \end{array}$$

- 5 In order to then obtain an uncorrected gene expression for *HER2-neu* from the TaqMan® instrument the following calculation is carried out:

$$\text{Uncorrected gene expression (UGE) for } HER2\text{-neu} = 2^{-\Delta C_t_{\text{test}}} / 2^{-\Delta C_t_{\text{calibrator}}}$$

- 10 *Normalizing UGE with known relative HER2-neu expression levels*

The normalization calculation entails a multiplication of the UGE with a correction factor ($K_{HER2\text{-neu}}$) specific to *HER2-neu* and a particular calibrator RNA. A correction factor $K_{HER2\text{-neu}}$ can also be determined for any internal control gene and any accurately pre-quantified calibrator RNA. Preferably, the internal control gene

15 β -actin and the accurately pre-quantified calibrator RNA, Human Liver Total RNA (Stratagene, Cat #735017) are used. Using β -actin and the accurately pre-quantified calibrator RNA, Human Liver Total RNA (Stratagene, Cat #735017) the correction factor $K_{HER2\text{-neu}}$ equals 12.6×10^{-3} .

- Normalization is accomplished using a modification of the ΔC_t method
- 20 described by Applied Biosystems, the TaqMan® manufacturer, in User Bulletin #2 and described above. To carry out this procedure, the UGE of 6 different FPE test tissues were analyzed for *HER2-neu* expression using the TaqMan® methodology described above. The internal control gene β -actin and the calibrator RNA, Human Liver Total RNA (Stratagene, Cat #735017) was used.

The already known relative *HER2-neu* expression level of each sample AG221, AG222, AG252, Adult Lung, PC3, AdCol is divided by its corresponding TaqMan® derived UGE to yield an unaveraged correction factor K.

$$K_{\text{unaveraged}} = \text{Known Values} / \text{UGE}$$

5

Next, all of the K values are averaged to determine a single K_{EGFR} correction factor specific for *HER2-neu*, Human Liver Total RNA (Stratagene, Cat #735017) calibrator, and β -actin.

Therefore, to determine the Corrected Relative *HER2-neu* Expression in an unknown tissue sample on a scale that is consistent with pre-TaqMan® *HER2-neu* expression studies, one merely multiplies the uncorrected gene expression data (UGE) derived from the TaqMan® apparatus with the $K_{HER2-neu}$ specific correction factor, given the use of the same internal control gene and calibrator RNA.

15 Corrected Relative *HER2-neu* Expression = UGE x $K_{HER2-neu}$

A $K_{HER2-neu}$ may be determined using any accurately pre-quantified calibrator RNA or internal control gene. Future sources of accurately pre-quantified RNA can be calibrated to samples with known relative *EGFR* expression levels as described in the method above or may now be calibrated against a previously calibrated calibrator RNA such as Human Liver Total RNA (Stratagene, Cat #735017) described above.

For example, if a subsequent $K_{HER2-neu}$ is determined for a different internal control gene and/or a different calibrator RNA, one should calibrate both the

internal control gene and the calibrator RNA to tissue samples for which *HER2-neu* expression levels relative to that particular internal control gene have already been determined or published. Such a determination can be made using standard pre-TaqMan®, quantitative RT-PCR techniques well known in the art. The known
5 expression levels for these samples will be divided by their corresponding UGE levels to determine a K for that sample. K values are then averaged depending on the number of known samples to determine a new $K_{HER2-neu}$ specific to the different internal control gene and/or calibrator RNA.

EXAMPLE 5

10 *Patient Population and Tissue Acquisition*

Patients. Eighty-three patients suffering from NSCLC consisting of sixty-five (78.3%) men and 18 (21.7%) women, with a median age of 63.5 years (range, 34-82) were studied. Thirty-nine (47%) patients had squamous cell carcinomas, 32 (38.6%) had adenocarcinoma, and 12 (14.5%) had large cell carcinomas. The
15 primary tumors were graded histopathologically as well-differentiated (G1, one patient), moderately-differentiated (G2, 18 patients), and poorly-differentiated (G3, 64 patients). Tumor staging was performed according to the International Union Against Cancer (UICC) TNM classification: Forty-one (49.4%) had stage I tumors, 16 (19.3%) had stage II tumors, and 26 (31.3%) had stage IIIa tumors. All tumors
20 were completely resected (R0 category), by at least a lobectomy as quality control. Patients with histopathological stage IIIa tumors received postoperative radiotherapy. The median follow-up was 85.9 months (min. 63.3; mar. 105.2 months) and no patient was lost to follow-up.

Tissue Acquisition. Tissue for gene expression analysis was obtained immediately after lung resection before starting mediastinal lymphadenectomy and was immediately frozen in liquid nitrogen. Tissues were analyzed from the following 2 locations: tumor and uninvolved lung tissue taken from the greatest distance to the tumor. 6 μ m frozen sections were taken from blocks of tumor tissue and starting with the first section every fifth was routinely stained with HE and histopathologically evaluated. Sections were pooled for analysis from areas of estimated 75% malignant cells. RNA was isolated from tissue samples according to the methods in Example 2.

10 EXAMPLE 6

Statistical Analysis

TaqMan® analyses yield values that are expressed as UGE. The ratio between UGE in tumor tissue and UGE in matching non-malignant lung tissue was used to determine differential gene expression. Associations between the two UGE variables were tested by using Wilcoxon signed rank test. The Chi-Square test was used to analyze the associations between categorical clinicopathological variables. Hazards ratios were used to calculate the relative risks of death. These calculations were based on the Pike estimate, with the use of the observed and expected number of events as calculated in the log-rank test statistic. Pike, J R Stat Soc Series A 135:201-203; 1972. The maximal chi-square method of Miller and Sigmund (Miller et al., Biometrics 38:1011-1016, 1982) and Halpern (Biometrics 38:1017-1023, 1982) was adapted to determine which expression value best segregated patients into poor- and good prognosis subgroups (in terms of likelihood of surviving), with the log-rank test as the statistics used to measure the strength of the grouping. To

determine a P value that would be interpreted as a measure of the strength of the association based on the maximal chi-square analysis, 1000 boot-strap-like simulations were used to estimate the distribution of the maximal chi-square statistics under the hypothesis of no association. Halpern, Biometrics 38:1017-1023, 1982. Cox's proportional hazards modeling of factors that were significant in anivariate analysis was performed to identify which factors might have a significant influence on survival. The level of significance was set to $p < 0.05$.

HER2-neu mRNA expression was detectable by quantitative real-time RT-PCR in 83 of 83 (100%) normal lung and 83 of 83 (100%) tumor samples. The corrected *HER2-neu* mRNA expression, expressed as the ratio between *HER2-neu* and β -Actin PCR product, was 4.17×10^{-3} (range 0.28 - 23.86×10^{-3}) in normal lung and 4.35×10^{-3} (range: 0.21 - 68.11×10^{-3}) in tumor tissue ($P=0.019$ Wilcoxon test). The maximal chi-square method by Miller and Siegmund (Miller et al., Biometrics 38:1011-1016, 1982) and Halpern (Biometrics 38:1017-1023, 1982) determined a threshold value of 1.8 to segregate patients into low and high differential *HER2-neu* expressors. By this criterion, 29 (34.9%) patients had a high differential *HER2-neu* expression and 54 (65.1 %) had a low differential *HER2-neu* expression. Figure 4 shows associations between clinicopathological data and differential *HER2-neu* gene expression status. There were no statistically significant differences detectable. Figure 1 displays a Kaplan Meier plot of the estimated probability of survival versus the differential *HER2-neu* mRNA expression status. The median survival was not reached in the low differential *HER2-neu* expression group compared to 31.1 months (95% C.I.: 21.96- 40.24) in the high differential *HER2-neu* expression group. To determine a P value, bootstrap-like simulations were used to estimate the

distribution of a maximal chi-square statistic, since the threshold value of 1.8 had been chosen after examining the data. The resulting adjusted *P* value was .004 (Log-rank test).

The accuracy of *HER2-neu* as a prognostic factor was next determined by the Cox's proportional hazards model analysis. In univariate analysis of potential prognostic factors, high differential *HER2-neu* expression as well as advanced pT (tumor stage) classification, pN (lymph node stage) classification, and tumor stage were significant unfavorable prognostic factors (Figure 5). In a multivariate analysis of prognostic factors (Figure 6), high differential *HER2-neu* expression was a significant and independent unfavorable prognostic factor, as well as advanced pN classification and tumor stage.

EGFR mRNA expression was detectable by quantitative real-time RT-PCR in 83 of 83 (100%) normal lung and 83 of 83 (100%) tumor samples. The median corrected *EGFR* mRNA expression was 8.17×10^{-3} (range: $0.31-46.26 \times 10^{-3}$) in normal lung and 7.22×10^{-3} (range: $0.27-97.49 \times 10^{-3}$) in tumor tissue ($P=n.s.$). The maximal chi-square method (Miller (1982); Halpern (1982)) determined a threshold value of 1.8 to segregate patients into low and high differential *EGFR* expressors. By this criterion, 28 (33.7%) patients had a high differential *EGFR* expression and 55 (66.3%) had a low differential *EGFR* expression status. There were no statistical significant differences between clinicopathological variables and differential *EGFR* mRNA expression status detectable (Figure 4). A trend towards inferior overall survival was observable for the high differential *EGFR* expression group, but did not reach statistical significance (Figure 2). The median survival was not reached in

the low differential *EGFR* expression group compared to 32.37 months (95% C.I.: 8.43-56.31) in the high differential *EGFR* expressor group ($P=0.176$).

High expression levels (above 1.8) of differential *HER2-neu* and *EGFR* were found in 14 of 83 (16.9%) patients. Forty of 83 (48.2%) patients showed a low differential expression status (below 1.8) for *HER2-neu* and *EGFR*, whereas 14 of 83 (16.9%) showed a high differential expression for *EGFR* only, and 15 of 83 (18.1%) patients displayed a high differential expression for *HER2-neu*. The median survival was not reached in the group that showed low differential *HER2-neu* and *EGFR* expression, compared to 45.47 months in the high differential *EGFR* expression group, 31.10 months (95% C.I.: 14.77-47.43) in the high differential *HER2-neu* expression group, and 22.03 months (95% C.I.: 2.30; 41.76; $P=0.003$; log-rank test; Figures 3 and 5) in the high differential *HER2-neu* and *EGFR* expression group. Univariate analysis displayed high differential *HER2-neu* and *EGFR* coexpression as a significant unfavorable prognostic factor (Figure 5). In a multivariate analysis of prognostic factors (Figure 6), high differential *HER2-neu* and high differential *EGFR* coexpression was a significant and independent unfavorable prognostic factor, as was advanced pN classification and tumor stage.

EXAMPLE 7

Tumor response to a receptor tyrosine kinase targeted chemotherapy

Five colon cancer patients' tumors were initially identified as expressing *EGFR* by immunohistochemistry. Patients were treated with Imclone IMC-C225, 400 mg/m² loading dose followed by 250 mg/m² weekly, plus CPT-11 at the same

dose and schedule that the patient had previously progressed on. Previous CPT-11 dose attenuations were maintained.

Using the methodology described in Examples 1-4, Patient 1 was determined to have a corrected EGFR expression level of 2.08×10^{-3} and had a completed
5 response (CR) to a a receptor tyrosine kinase targeted chemotherapy comprising CPT-11 (7-ethyl-10-[4-(1-piperidino)-1-piperidino] carboxycamptothecin) / C225 (an anti- EGFR monoclonal antibody effective in anti-cancer therapy; Mendelsohn, Endocr Relat Cancer 2001 Mar;8(1):3-9). Patient 2 had a corrected EGFR expression level of 8.04×10^{-3} and had a partial response (PR) to the receptor
10 tyrosine kinase targeted chemotherapy. Patient 3 had a corrected EGFR expression level of 1.47×10^{-3} and also showed a partial response (PR) to the receptor tyrosine kinase targeted chemotherapy. Patient 4 had a corrected EGFR expression level of 0.16×10^{-3} and had stable disease (SD) showing no response to the receptor tyrosine kinase targeted chemotherapy. Patient 5 had a no EGFR expression (0.0×10^{-3}) and had progressive disease (PR) showing no response to the receptor tyrosine
15 kinase targeted chemotherapy. See figure 9.

What is claimed is:

1. A method for determining a chemotherapeutic regimen comprising receptor tyrosine kinase targeted agent, for treating a tumor in a patient comprising:
 - (a) obtaining a tissue sample of the tumor;
 - 5 (b) obtaining a non-malignant tissue sample matching said tumor;
 - (c) isolating mRNA from the tumor sample and non-malignant sample;
 - (d) subjecting the mRNA to amplification using a pair of oligonucleotide primers that hybridize under stringent conditions to a region of the *EGFR* gene, or a pair of oligonucleotide primers that hybridize under stringent conditions to a region of the *HER2-neu* gene, to obtain an *EGFR* tumor amplified sample and a *EGFR* non-malignant amplified sample, or a *HER2-neu* tumor amplified sample and a *HER2-neu* non-malignant amplified sample
 - 10 (e) determining the amount of *HER2-neu* mRNA in the *HER2-neu* tumor amplified sample and *HER2-neu* non-malignant amplified sample or determining the amount of *EGFR* mRNA in the *EGFR* tumor amplified sample and *EGFR* non-malignant amplified sample;
 - (f) obtaining a differential *HER2-neu* expression level or obtaining a differential *EGFR* expression level; and
 - 15 (g) determining a chemotherapeutic regimen comprising a receptor tyrosine kinase targeted agent by comparing the differential *HER2-neu* expression level and the threshold level for *HER2-neu* gene expression, or comparing the differential *EGFR* expression level and the threshold level for *EGFR* gene expression.
- 25 2. A method for determining a chemotherapeutic regimen receptor tyrosine kinase targeted agent for treating a tumor in a patient comprising:
 - (a) obtaining a tissue sample of the tumor;
 - (b) obtaining a non-malignant tissue sample matching said tumor;
 - (c) isolating mRNA from the tumor sample and non-malignant sample;
 - 30 (d) subjecting the mRNA to amplification using a pair of oligonucleotide primers that hybridize under stringent conditions to a region of the *EGFR* gene, to obtain an tumor amplified sample and a non-malignant amplified sample;
 - (e) determining the amount of *EGFR* mRNA in the tumor amplified sample and non-malignant amplified sample;
 - 35 (f) obtaining a differential *EGFR* expression level; and
 - (g) determining a chemotherapeutic regimen comprising a receptor tyrosine kinase targeted by comparing the differential *EGFR* expression level and the threshold level for *EGFR* gene expression.

3. A method for determining a chemotherapeutic regimen comprising receptor tyrosine kinase targeted agent for treating a tumor in a patient comprising:
 - (a) obtaining a tissue sample of the tumor;
 - (b) obtaining a non-malignant tissue sample matching said tumor;
 - 5 (c) isolating mRNA from the tumor sample and non-malignant sample;
 - (d) subjecting the mRNA to amplification using a pair of oligonucleotide primers that hybridize under stringent conditions to a region of the *HER2-neu* gene, to obtain an tumor amplified sample and a non-malignant amplified sample;
 - 10 (e) determining the amount of *HER2-neu* mRNA in the tumor amplified sample and non-malignant amplified sample;
 - (f) obtaining a differential *HER2-neu* expression level; and
 - (g) determining a chemotherapeutic regimen comprising a receptor tyrosine kinase targeted agent by comparing the differential *HER2-neu* expression level and the threshold level for *HER2-neu* gene expression.
 - 15
4. A method for determining a chemotherapeutic regimen comprising receptor tyrosine kinase targeted agent for treating a tumor in a patient comprising:
 - (a) obtaining a tissue sample of the tumor;
 - 20 (b) obtaining a non-malignant tissue sample matching said tumor;
 - (c) isolating mRNA from the tumor sample and non-malignant sample;
 - (d) subjecting the mRNA to amplification using a pair of oligonucleotide primers that hybridize under stringent conditions to a region of the *EGFR* gene, and a pair of oligonucleotide primers that hybridize under stringent conditions to a region of the *HER2-neu* gene, to obtain an *EGFR* tumor amplified sample and an *EGFR* non-malignant amplified sample, and a *HER2-neu* tumor amplified sample and a *HER2-neu* non-malignant amplified sample
 - 25
 - (e) determining the amount of *HER2-neu* mRNA in the *HER2-neu* tumor amplified sample and *HER2-neu* non-malignant amplified sample and determining the amount of *EGFR* mRNA in the *EGFR* tumor amplified sample and *EGFR* non-malignant amplified sample;
 - 30
 - (f) obtaining a differential *HER2-neu* expression level and obtaining a differential *EGFR* expression level; and
 - 35
 - (g) determining a chemotherapeutic regimen comprising a receptor tyrosine kinase targeted agent by comparing the differential *HER2-neu* expression level and the threshold level for *HER2-neu* gene expression, and comparing the differential *EGFR* expression level and the threshold level for *EGFR* gene expression.
- 40 5. The method claim 2, wherein the oligonucleotide primers consist of the oligonucleotide primer pair *EGFR*, or pair of oligonucleotide primers substantially identical thereto.

6. The method of claim 3 wherein the oligonucleotide primers consist of the oligonucleotide primer pair HER2-neu, or pair of oligonucleotide primers substantially identical thereto.
7. The method of any one of claims 1, 2, 3, or 4 wherein the tumor is a non-small cell lung cancer tumor.
8. The method of claim 4 wherein the primers consist of both the oligonucleotide primer pair HER2-neu and oligonucleotide primer pair EGFR.
9. The method of any one of claims 1, 2, or 4 wherein the threshold level of *EGFR* gene expression is about 1.8 times *EGFR* gene expression in matching non-malignant tissue.
10. The method of any one of claims 1, 3, or 4 wherein, the threshold level of *HER2-neu* gene expression is about 1.8 times *HER2-neu* gene expression in matching non-malignant tissue.
11. The method of any one of claims 1, 2, 3 or 4 wherein the tissue samples are are fixed or fixed and paraffin embedded.
12. A method for determining the level of *EGFR* expression in a fixed paraffin embedded tissue sample comprising;
 - (a) deparaffinizing the tissue sample, to obtain a deparaffinized sample;
 - (b) isolating mRNA from the deparaffinized sample in the presence of an effective amount of a chaotropic agent;
 - (c) subjecting the mRNA to amplification using a pair of oligonucleotide primers that hybridize under stringent conditions to a region of the *EGFR* gene, to obtain an amplified sample;
 - (d) determining the quantity of *EGFR* mRNA relative to the quantity of mRNA of an internal control gene.
13. The method of claim 12 wherein, the pair of oligonucleotide primers consists of the oligonucleotide primer pair EGFR or a pair of oligonucleotide primers substantially similar thereto.
14. A method for determining the level of *HER2-neu* expression in a fixed paraffin embedded tissue sample comprising;
 - (a) deparaffinizing the tissue sample, to obtain a deparaffinized sample;
 - (b) isolating mRNA from the deparaffinized sample in the presence of an effective amount of a chaotropic agent;

- (c) subjecting the mRNA to amplification using a pair of oligonucleotide primers that hybridize under stringent conditions to a region of the *HER2-neu* gene, to obtain an amplified sample;
 - (d) determining the quantity of *HER2-neu* mRNA relative to the quantity of mRNA of an internal control gene.
- 15. The method of claim 14 wherein, the pair of oligonucleotide primers consists of the oligonucleotide primer pair *HER2-neu* or a pair of oligonucleotide primers substantially similar thereto.
- 16. The method of claim 12 or 14 wherein the internal control gene is β -actin.
- 10 17. The method of claim 12 or 14 wherein, mRNA isolation is carried out by
 - (a) heating the tissue sample in a solution comprising an effective concentration of a chaotropic compound to a temperature in the range of about 75 to about 100 °C for a time period of about 5 to about 120 minutes;
 - 15 and
 - (b) recovering said mRNA from the chaotropic solution.
- 18. An oligonucleotide primer having the sequence of SEQ ID NO: 1 or and an oligonucleotide substantially identical thereto.
- 20 19. An oligonucleotide primer having the sequence of SEQ ID NO: 2 or and an oligonucleotide substantially identical thereto.
- 20. An oligonucleotide primer having the sequence of SEQ ID NO: 4 or and an oligonucleotide substantially identical thereto.
- 21. An oligonucleotide primer having the sequence of SEQ ID NO: 5 or and an oligonucleotide substantially identical thereto.
- 25 22. A kit for detecting expression of an *EGFR* gene comprising oligonucleotide pair *EGFR* or an oligonucleotide pair substantially identical thereto.
- 23. A kit for detecting expression of a *HER2-neu* gene comprising oligonucleotide pair *HER2-neu* or an oligonucleotide pair substantially identical thereto.
- 30 24. A kit for detecting expression of a *HER2-neu* and *EGFR* gene comprising oligonucleotide pair *HER2-neu* or an oligonucleotide pair substantially identical thereto and oligonucleotide pair *EGFR* or an oligonucleotide pair substantially identical thereto.

Figure 1

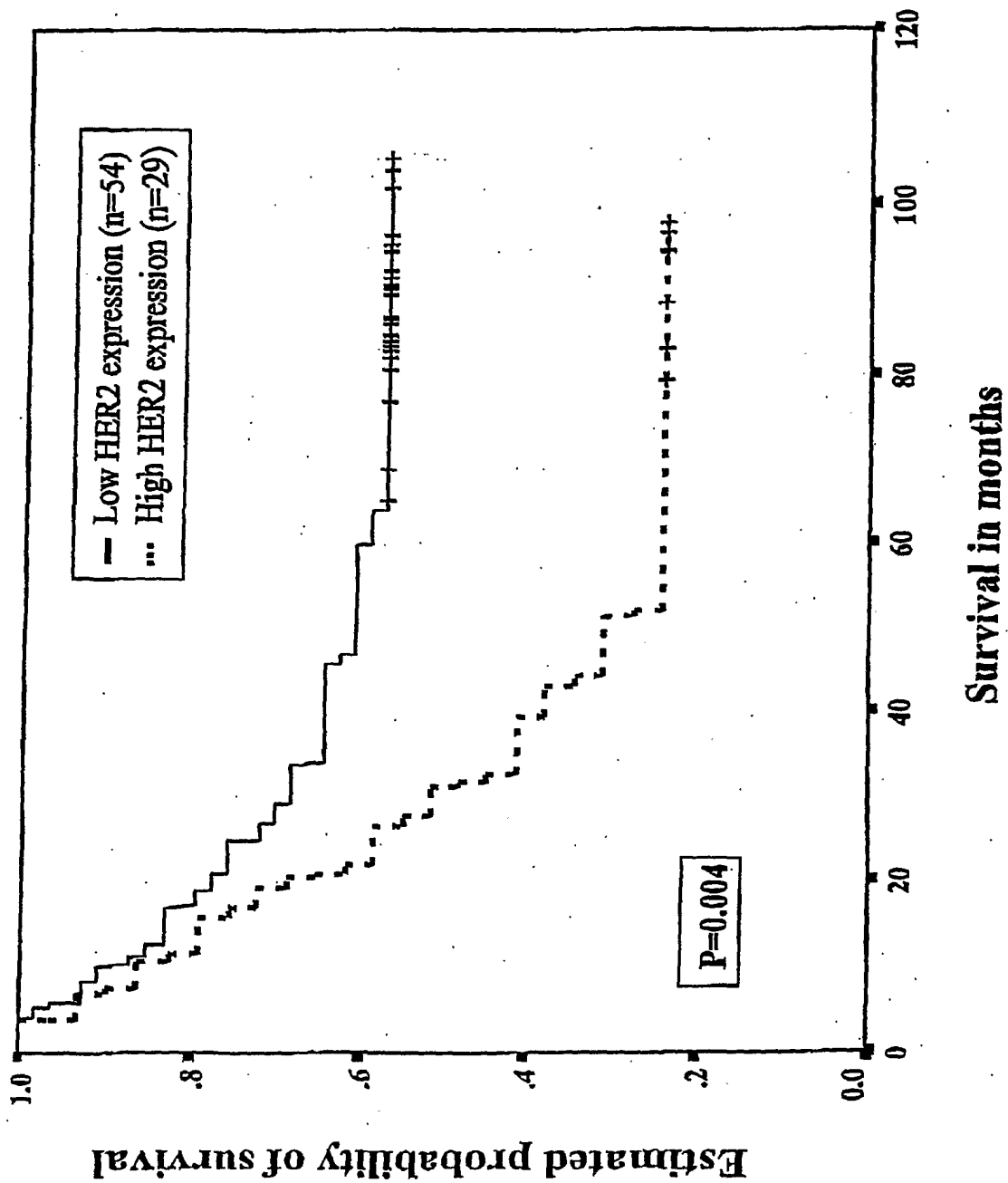


Figure 2

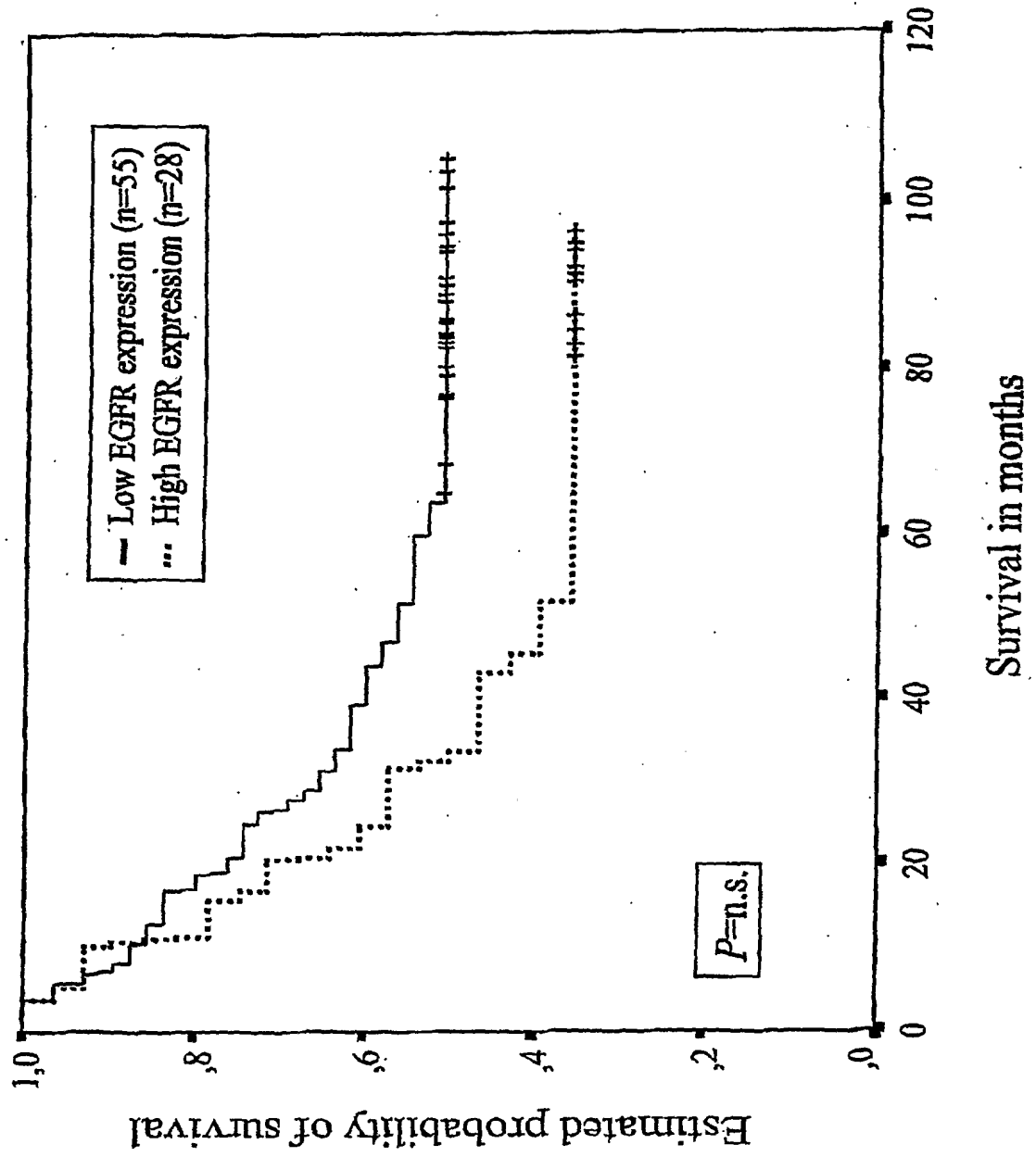


Figure 3

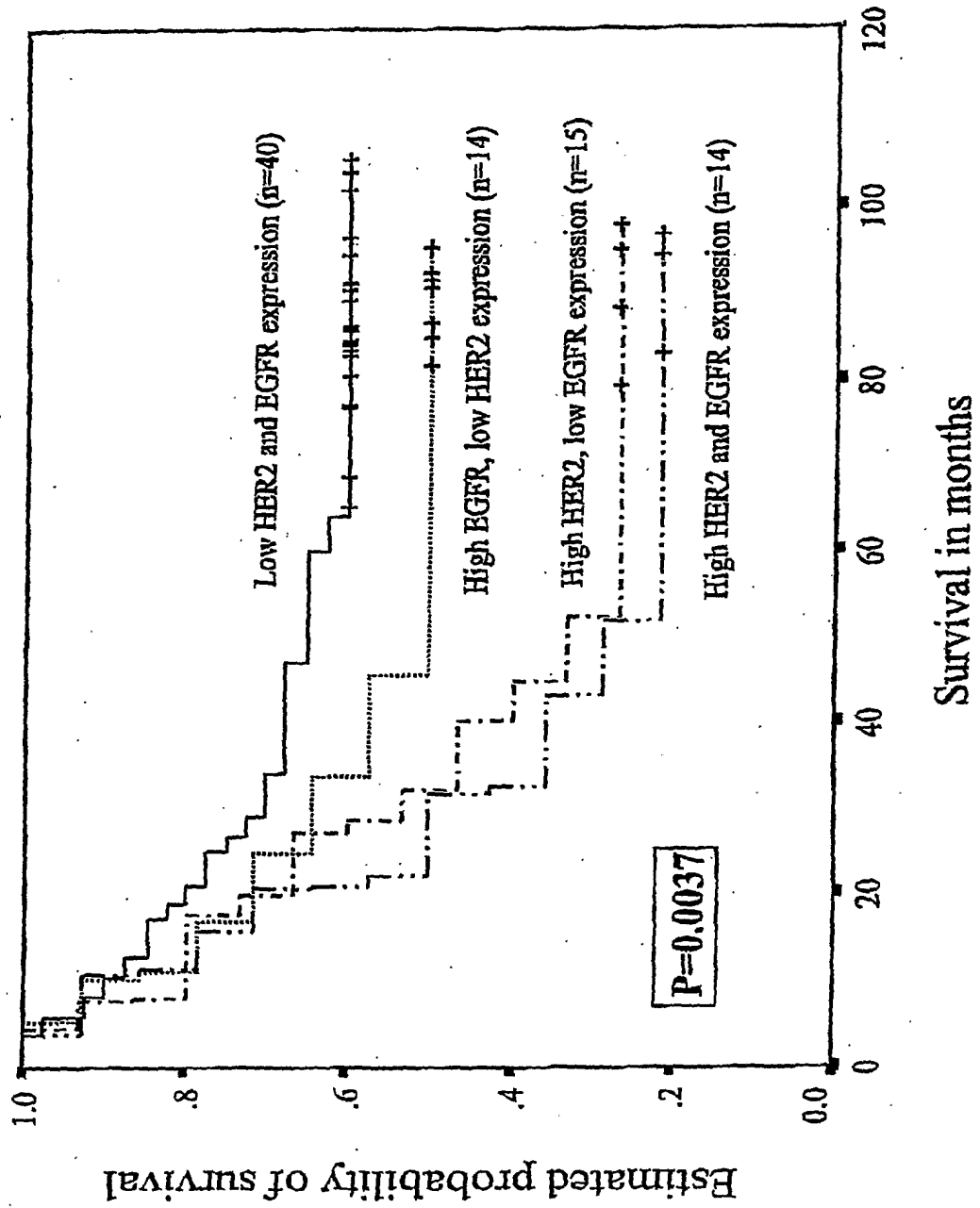


Figure 4 Relationships with HER2 neu and EGFR mRNA expression

Variable	EGFR			HER2 neu		
	low	high	P	low	high	P
Age	40	25		45	20	
Sex	15	3	0.84	20	9	0.13
Smoker	48	25		46	27	
Smoker category	7	3	0.79	8	2	0.29
Stage	11	5		12	4	
Stage category	35	19		33	21	
	9	4	0.93	9	4	0.56
pN1	31	15		32	14	
pN2	15	7		15	7	
UICC Stage	9	6	0.85	7	8	0.25
I	26	15		28	13	
II	13	3		12	4	
IIIa	16	10	0.37	14	12	0.31
Histology						
Squamous Cell Carcinoma	26	13		28	11	
Adenocarcinoma	20	12		16	16	
Large Cell Carcinoma	9	3	0.74	10	2	0.57
Grading						
Well differentiated	1	0		1	0	
Moderately differentiated	13	5		13	5	
Poorly differentiated	41	23	0.63	40	24	0.57

Figure 5 Survival in NSCLC based on clinical and molecular parameters

Parameter		n	5-year survival (%) ± SD	Median survival (months)	CI 95%	P value
Stage	I	41	68.2 ± 0.07	n.r.	-	<0.0001
	II	16	43.8 ± 0.12	33.97 ± 5.70	22.80; 45.14	
	IIIA	26	11.5 ± 0.06	19.00 ± 5.14	8.92; 29.08	
	pT ₁	16	68.8 ± 0.12	n.r.	-	0.0157
	pT ₂	34	44.4 ± 0.07	46.77 ± 18.51	10.49; 83.05	
	pT ₃	26	23.1 ± 0.12	26.67 ± 6.09	14.73; 38.61	
	pN ₀	44	67.3 ± 0.07	n.r.	-	<0.0001
	pN ₁	22	31.8 ± 0.10	33.71 ± 6.86	20.22; 47.12	
	pN ₂	15	0	16.70 ± 4.01	8.84; 24.56	
Tumor	Low	54	57.4 ± 0.07	n.r.	-	0.0044
	High	29	24.1 ± 0.08	31.10 ± 4.66	21.96; 40.24	
	Low	55	50.8 ± 0.07	n.r.	-	n.s.
	High	28	35.7 ± 0.09	32.37 ± 12.22	8.43; 56.31	
Marker	Double low	40	59.9 ± 0.08	n.r.	-	0.0037
	EGFR high	14	50.0 ± 0.13	45.47	-	
	HER-2 high	15	26.7 ± 0.11	31.10 ± 8.33	-14.77; 47.73	
	Double high	14	21.4 ± 0.11	22.03 ± 10.07	2.30; 41.76	

Abbreviations: n.r. (not reached); - (cannot be calculated); CI 95% (95% confidence interval); n.s. (not significant); n (number of patients).

Figure 6. Cox-proportional hazard regression models

Model	Parameter	Hazards ratio	CI 95%	P value
D	Stage			0.0001
	I/IIIa	0.219	0.11-0.44	0.0001
	II/IIIa	0.524	0.23-1.17	0.177
	HER2-neu	1.894	1.02-3.51	0.043
	pT			0.127
	pT ₁ /pT ₃	0.311	0.10-0.97	0.044
	pT ₂ /pT ₃	0.692	0.32-1.50	0.354
	pN			0.0001
	pN ₀ /pN ₂	0.143	0.07-0.31	0.0001
	pN ₁ /pN ₂	0.333	0.14-0.75	0.008
	HER2-neu	1.890	1.03-3.48	0.041
	Stage			0.0001
	I/IIIa	0.554	0.11-0.44	0.0001
	II/IIIa	0.554	0.24-1.26	0.159
	Double marker	1.331	1.03-1.73	0.03
	pT			0.168
	pT ₁ /pT ₃	0.335	0.11-1.05	0.061
	pT ₂ /pT ₃	0.704	0.32-1.55	0.384
	pN			0.0001
	pN ₀ /pN ₂	0.143	0.07-0.31	0.0001
	pN ₁ /pN ₂	0.143	0.14-0.74	0.007
	Double marker	1.280	1.00-1.63	0.046

Abbreviations: CI 95% (confidence interval for hazards ratio); Parameter section: e.g. stage I/IIIa means stage I compared to stage IIIa.

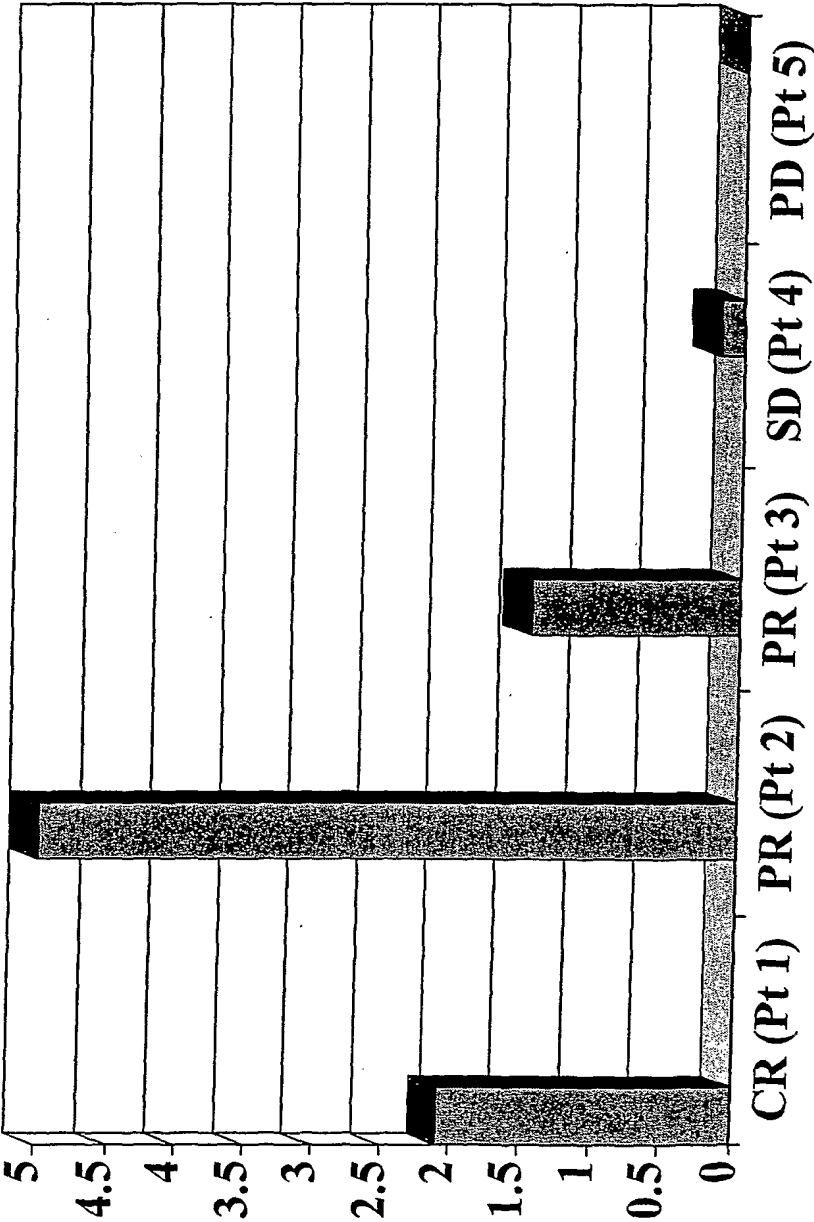
Figure 7: Chart illustrating how to calculate *EGFR* expression relativ

	from "test" reactions				from "calibration" reactions				Uncorrected Gene Expression (USE)	Known <i>EGFR</i> values	Derivation of K_{EGFR} (Average K)		Relative <i>EGFR</i> exp.
	Sample	C_T <i>EGFR</i>	C_T β -actin	ΔC_T	$2^{-\Delta C_T}$	C_T <i>EGFR</i>	C_T β -actin	ΔC_T			K	K_{EGFR}	
Experimental	unknown 1	32.7	26.8	5.9	0.0167	-	-	-	0.525	-	-	26.95×10^3	14.4×10^3
	unknown 2	32.88	26.43	6.45	0.0114	-	-	-	0.358	-	-	26.95×10^3	9.66×10^3
	Calib. RNA	-	-	-	-	27.01	22.04	4.97	$0.0319/0.0319 = 1$	-	-	-	-
From Published Data													
	60N	31.61	23.86	7.75	0.00464	-	-	-	0.2117	5.70×10^3	26.95×10^3	26.95×10^3	-
	60T	29.08	20.65	8.43	0.0029	-	-	-	0.1321	3.56×10^3	26.95×10^3	26.95×10^3	-
	SF12A	28.71	20.76	7.95	0.0040	-	-	-	0.184	4.97×10^3	26.95×10^3	26.95×10^3	-
	SF12B	24.69	19.87	4.82	0.0354	-	-	-	1.613	43.5×10^3	26.95×10^3	26.95×10^3	-
	Cir11	24.03	16.3	7.73	0.0047	-	-	-	0.215	5.78×10^3	26.95×10^3	26.95×10^3	-
	AdCol	26.04	17.06	8.98	0.00198	-	-	-	0.080	2.43×10^3	26.95×10^3	26.95×10^3	-
	Calib. RNA	-	-	-	-	25.96	18.57	7.39	$0.00596/0.00596 = 1$	-	-	-	-

Figure 8: Chart illustrating how to calculate *HER2-neu* expression relative to an internal control gene

	from "test" reactions				from "calibration" reactions				Uncorrected Gene Expression (UGE)	Known <i>HER2-neu</i> values	Derivation of <i>K_{HER2-neu}</i> (average K)	Relative <i>HER2-neu</i> exp
	Sample	<i>C_T</i> <i>HER2-neu</i>	<i>C_T</i> <i>β-actin</i>	ΔC_T	$2^{-\Delta C_T}$	<i>C_T</i> <i>HER2-neu</i>	<i>C_T</i> <i>β-actin</i>	ΔC_T	$2^{-\Delta C_T}$			
Experimental	unknown 1	21.5	16.3	5.2	0.0272	-	-	-	1.43	-	13.3×10^3	19.1×10^3
	unknown 2	23.22	17.05	6.16	0.0139	-	-	-	0.74	-	13.3×10^3	9.8×10^3
	Calib. RNA	-	-	-	-	24.29	18.57	5.72	0.0189	0.0189/0.0189 = 1	-	-
From Published Data	60N	30.28	23.86	6.42	0.012	-	-	-	0.702	9.34×10^3	13.3×10^3	-
	60T	27.67	20.65	7.22	0.0067	-	-	-	0.403	5.36×10^3	13.3×10^3	-
	SF12A	25.01	20.76	4.25	0.0525	-	-	-	3.16	42.03×10^3	13.3×10^3	-
	SF12B	26.07	19.87	6.2	0.0136	-	-	-	0.817	10.88×10^3	13.3×10^3	-
	Clr11	21.5	16.3	5.2	0.0272	-	-	-	1.635	21.76×10^3	13.3×10^3	-
	AdCol	23.22	17.06	6.16	0.014	-	-	-	0.841	11.18×10^3	13.3×10^3	-
	Calib. RNA	-	-	-	-	25.0	19.09	5.91	0.0166	0.0166/ 0.0166 = 1	-	-

Fig. 9: CPT-11/C 225 Mediated Tumor Response and EGF-R Gene Expression



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